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<p>(54) Title: ANTI-HIV PROTEIN (57) Abstract The use of the poxvirus 35 kDa major secretory protein or a related protein or a biologically active fragment thereof, as an anti-viral agent in particular for inhibiting infection of target cells by HIV.</p>		

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## ANTI-HIV PROTEIN

This invention relates to chemokine-binding proteins, in particular poxvirus chemokine-binding proteins, having anti-viral properties.

- 5 The invention also relates to a variety of uses of the chemokine-binding proteins including anti-viral therapy.

Chemokines are a family of small secreted polypeptides. They are chemoattractant cytokines that direct migration of immune cells into sites of inflammation and are important in defence and control of

10 infectious agents such as HIV (D'Souza & Harden, 1996; Fauci, 1996; Howard *et al.*, 1996; Murphy, 1996; Premack & Schall, 1996). Chemokines are usually between 80-100 amino acids in length and have a particular pattern of cysteine amino acid residues which characterise them as chemokines. More than 30 different chemokines have been identified and

15 these are divided into at least three structural groups based on the number and arrangement of conserved cysteines: CC ( $\beta$ ) chemokines such as RANTES, macrophage inflammatory protein (MIP)-1 $\alpha$  and eotaxin; CXC ( $\alpha$ ) chemokines such as IL-8 and growth-related oncogene (GRO)- $\alpha$  (alternatively known as melanoma growth stimulating activity [MGSA]); and

20 the C chemokine lymphotactin. These different chemokines have evolved to function with particular cell types. For example, most CC chemokines are chemoattractants for monocytes, while most CXC chemokines are chemoattractants for neutrophils. However, there is no rule relating chemokine structure and cellular specificity to other leukocyte subtypes.

25 The C chemokine lymphotactin is selective for T cells and the CC chemokine eotaxin is highly specific for eosinophils.

Chemokines exert their activity by interacting with seven-transmembrane-domain chemokine receptors (CKRs) expressed in different cell subsets (Howard *et al.*, 1996; Murphy, 1996; Premack &

30 Schall, 1996), thus determining the leukocyte subtype that predominates in

different types of inflammation. Chemokines are thought to form a chemical gradient in an immobilised phase via electrostatic interactions with negatively charged proteoglycans, which may increase the specificity of chemokine action (Schall & Bacon, 1994; Witt & Lander, 1994). There is  
5 evidence that the binding site of chemokines for proteoglycans and specific CKRs are distinct (Webb *et al.*, 1993; Graham *et al.*, 1996).

The activity of chemokines is tightly regulated to prevent excessive inflammation that can cause disease, and these molecules represent potential targets for therapeutic intervention in a wide range of  
10 diseases. The production of soluble versions of cytokine receptors containing only the extracellular binding domain represents a physiological and therapeutic strategy to block the activity of some cytokines (Rose-John & Heinrich, 1994; Heaney & Golde, 1996). However, the seven transmembrane domain structure of CKRs makes the construction of  
15 soluble, inhibitory CKRs difficult, and thus antagonists based on mutated chemokines, blocking peptides or antibodies are alternative inhibitors of chemokines under evaluation (D'Souza & Harden, 1996; Howard *et al.*, 1996).

Poxviruses, a family of complex DNA viruses (Moss, 1996),  
20 have evolved unique strategies for evasion of the host immune response (Alcami & Smith, 1995a; McFadden *et al.*, 1995; Smith, 1996; Spriggs, 1996) and are the only virus family known to produce secreted versions of receptors for cytokines such as TNF, IL-1 $\beta$ , IFN- $\alpha/\beta$  and IFN- $\gamma$ . These poxvirus proteins bind cytokines with high affinity and block their activity by  
25 preventing interaction with receptors on the target cell. Inactivation of virus genes encoding these cytokine inhibitors has profound effects on viral pathogenesis. Poxvirus cytokine receptors were initially identified from sequence similarity to the extracellular binding domain of cellular cytokine receptors, but binding and functional analysis led to the identification of the  
30 IFN- $\alpha/\beta$ R encoded by vaccinia virus (VV), which has very limited similarity

to known cellular counterparts (Colamonici *et al.*, 1995; Symons *et al.*, 1995).

CKRs play a critical role in the transmission and dissemination of HIV by acting as a cofactor which is required with CD4 for virus entry and infection (D'Souza & Harden, 1996; Fauci, 1996; Berger, 1997). The CXCR4 CKR is a cofactor for T cell-tropic HIV isolates, whereas the CCR5 CKR is used by macrophage-tropic HIV strains. The importance of CCR5 *in vivo* is illustrated by the finding that individuals who are homozygous for a mutant version of the CCR5 gene are resistant to HIV infection. The chemokine receptors CCR5, CCR4, CCR3 and CCR2 have all been shown to mediate HIV entry into macrophages. Binding of chemokines or mutated chemokine antagonists to CKRs block HIV infection, illustrating the potential of blockade of HIV-CKR interaction as a preventative and therapeutic strategy against HIV (D'Souza & Harden, 1996; Fauci, 1996; Berger, 1997).

The malarial parasite *Plasmodium vivax* uses a CKR of unknown function (Duffy antigen) to enter and infect erythrocytes (Horuk *et al.*, 1993). The lack of known signalling function of the Duffy antigen and its expression in erythrocytes led to the suggestion that it could act as a sink for chemokines in the blood. Similar to HIV infections, binding of chemokines via the Duffy antigen blocks infection of erythrocytes by *P. vivax*, and individuals who lack the Duffy antigen on their red cells are resistant to *P. vivax* malaria. Thus, blockade of the interaction of the malarial parasite with the Duffy antigen is a potential target for intervention and development of drugs for *P. vivax*.

WO 96/33730 describes a chemokine binding protein designated CBP-I, which is the product of the T7 gene of myxoma virus. Myxoma virus T7 protein had previously been shown to function as a soluble interferon- $\gamma$  receptor. Proteins similar to CBP-I in other

leporipoxviruses (e.g. Shope fibroma virus), but not in the orthopoxviruses tested, were also found to bind chemokines.

WO/9711714 describes a chemokine-binding protein designated p35, which is derived from cowpox virus or VV strain Lister or from variola virus. P35 binds chemokines which are members of the CC branch of chemokines.

It is now been discovered that the 35 kDa major secretory protein encoded by VV and other poxviruses, which corresponds to the p35 protein of WO 97/11714 and is also termed vCKBP, is not only a chemokine-binding protein but it is also capable of inhibiting infection of cells by HIV.

The sequence of the gene encoding the secreted 35 kDa protein of VV Lister can be found in DNA sequence database of Japan, accession number D00612, and is shown in Fig. 18. The amino acid sequence of the protein encoded by this gene is shown in Fig. 19. The corresponding gene sequence for VV strain Copenhagen has accession number M35027 (GenBank). Sequences for the related VV gene A41 from Copenhagen and Western Reserve (WR) strains are given in M35027 (GenBank) and D11079 (DNA database of Japan), respectively.

The invention therefore provides in one aspect a method for inhibiting infection of a target cell by a virus, which normally infects the target cell by binding to a CKR on the target cell, which method comprises contacting the target cell and solution containing the virus with an effective amount of an anti-viral agent which is a protein from the 35 kDa major secretory protein family or a biologically active fragment thereof.

In another aspect, the invention provides the use of a protein from the 35 kDa major secretory protein family or a biologically active fragment thereof or a nucleic acid encoding the protein or fragment, together with a pharmaceutically acceptable carrier, in the manufacture of a medicament for treatment or prophylaxis of a viral infection.

In yet another aspect the invention provides a medicament, comprising a recombinant poxvirus derived from a poxvirus which naturally encodes a chemokine-binding protein which is a member of the poxvirus 35 kDa major secreted protein family, the recombinant poxvirus genetically engineered to be incapable of expressing the functional chemokine-binding protein. Such a medicament will provide poxvirus-based vaccines which are safer and more immunogenic than existing poxvirus vaccines.

The 35 kDa protein of poxviruses such as VV Lister is from a family of related chemokine-binding proteins with shared characteristics that may be defined as follows:

- conserved cysteine residues in the amino acid sequence which form intramolecular disulphide bonds. For example, the 35 kDa protein of VV strain Lister shares 8 cysteine residues in common with the 35K protein of cowpox virus and variola virus as described in WO 97/11714, the T1 protein of Shope fibroma virus, and with the VV strain WR A41 protein, out of a total of 8 cysteine residues downstream of the signal peptide: these residues align perfectly (Fig. 8).
- Chemokine-binding properties, though not necessarily with respect to the same chemokine or range of chemokines, or affinity constant for these chemokines.
- similar size. For examples the 35 kDa protein of VV strain Lister is made up of an amino acid sequence 258 residues long, and the comparable protein from variola virus strain India 1967 is 253 amino acids long, the Shope fibroma virus T1 protein is 252 amino acids, the VV strain WR A41 protein is 219 amino acids, and the A41 protein from variola virus strains Harvey or India 1967 are 218 amino acids (Fig. 8).
- amino acid identity /similarity. There is 85% identity between the amino acid sequences of the 35 kDa protein of VV strain Lister and the p35 protein of cowpox virus, and 95% amino acid identity between the 35 kDa protein of VV strain Lister and the p35 protein of variola virus. There is

also 24% identity and 49% similarity between the 35 kDa protein of VV strain Lister and the A41L protein of VV strain WR (Fig. 8). Amino acid similarity means amino acid residues having similar properties such as charge and hydrophobicity: where one amino acid maybe conservatively substituted (that is without significant effect on the structure of the protein) for another, there is "similarity" between the two.

- acidity; the proteins are all reasonably acidic. This is consistent with their ability to bind chemokines which tend to be basic proteins. The isoelectric points of the 35 kDa VV strain Lister protein and the A41L protein from VV strain WR are 4.2 and 5.4 respectively.

As well as poxvirus proteins, the 35 kDa protein family may include proteins from cellular sources, in particular mammalian cells. Numerous proteins represent a viral version of a protein from a mammalian host. The identification of known or newly-discovered proteins as being members of a family such as the 35 kDa protein family described herein is within the capabilities of the skilled artisan.

Figure 8 shows an amino acid sequence alignment between the VV strains WR (Smith *et al.*, 1991) and Copenhagen (Goebel *et al.*, 1990) A41L proteins, the comparable proteins from variola virus strains Harvey (Aguado *et al.*, 1992) and India 1967 (Shchelkunov *et al.*, 1994), the 35 kDa protein from VV strain Lister (Patel *et al.*, 1990), cowpox virus (Hu *et al.*, 1994), and variola virus strain India 1967 (Shchelkunov *et al.*, 1994), and the Shope fibroma virus T1 protein (Upton *et al.*, 1987). The proteins are clearly related and the size difference is mainly due to the additional sequences at one position in the 35 kDa proteins.

The relationship between some of the known proteins in this family has been established previously, for example between A41L from VV strain WR and the T1 protein of Shope fibroma virus (Howard *et al.*, 1991) (in that reference A41L was referred to as SalF4L), and the 35 kDa protein of VV strain Lister (Martinez-Pomares *et al.*, 1995).



The 35 kDa VV strain Lister protein and related proteins for use in the invention may be provided in native or in mutant form for use in the invention. Known methods, in particular genetic manipulation techniques, can be used to provide modified versions of the protein which  
5 have altered characteristics compared to the native proteins. Desirable altered characteristics may be for example an altered binding capability to enhance binding and therefore inhibition of viral infection.

The invention also envisages the use of fragments of the 35 kDa protein and related proteins, which fragments have anti-viral  
10 properties. The fragments may be peptides derived from the protein. Use of such peptides can be preferable to the use of an entire protein or a substantial part of the protein, for example because of the reduced immunogenicity of a peptide compared to a protein. Such peptides may be prepared by a variety of techniques including recombinant DNA techniques  
15 and synthetic chemical methods.

It will also be evident that the anti-viral proteins for use in the invention may be prepared in a variety of ways, in particular as recombinant proteins in a variety of expression systems.

The mechanism for inhibition of HIV infection by the 35 kDa  
20 protein (vCKBP) is believed to be binding of the protein to one or more CC chemokines which is/are required for virus replication in macrophages. The evidence so far suggests that the mechanism does not involve binding of the inhibitory protein to the HIV envelope (which might have been predicted, since gp120 normally binds the CKR when HIV infects a cell),  
25 although it is still possible that such an interaction can take place. A demonstration of these findings is shown in the examples contained herein. A particular chemokine-binding protein will inhibit infection by HIV strains which infect their target cells via the relevant CKR. Thus, chemokine-binding proteins which bind to CC chemokines can be expected to inhibit  
30 infection by viruses which use the CCR5 CKR as a co-receptor, found on

macrophages and monocytes. CC chemokine-binding proteins will be effective against macrophage tropic strain HIV strains.

In the attached figures:

Figures 1 to 7 show chemokine-binding properties of the 35 kDa protein.

Figure 8 shows an alignment of amino acid sequences between proteins in the 35 kDa family.

Figures 9 and 10 show anti-HIV properties of the 35 kDa protein.

Figures 11 and 12 illustrate models for blocking of HIV infection by the 35 kDa protein.

Figures 13 to 16 further illustrate inhibition of HIV infection by the 35 kDa protein.

Figures 17 and 18 show the DNA sequence and amino acid sequence for the 35 kDa secretory protein of VV strain Lister, respectively.

Sequences for proteins in the 35 kDa family are located in databases as follows (some of these amino acid sequences appear in Figure 8):

	A41L vaccinia WR	D11079
20	A41L vaccinia Copenhagen	M35027
	A41L variola Harvey	JQ1847
	A41L variola India-1967	X69198
	A41L variola Bangladesh-1975	L22579
25	35K vaccinia Lister	D00612
	35K cowpox	L08906
	35K variola India-1967	X69198
	35K variola Bangladesh-1975	L22579
	35K Shope fibroma T1	A43692

## EXAMPLES

### Example 1

#### Chemokine binding properties of 35 kDa protein (vCKBP).

#### 5 **Materials and Methods**

##### **Cells and viruses**

The growth conditions of human U937 and TK-143B cells, and the sources of VV strains have been described (Alcami & Smith, 1995b). The Lister strain used in this study and a Lister virus lacking the  
10 gene encoding the 35 kDa protein (Lister $\Delta$ 35K or V357) (Patel *et al.*, 1990) were kindly provided by A. H. Patel (Institute of Virology, Glasgow, UK).

##### **Reagents**

Radioiodinated recombinant human IFN- $\gamma$  (90  $\mu$ Ci/ $\mu$ g) and  
15 RANTES, MIP-1 $\alpha$ , IL-8 and GRO- $\alpha$  (2200 Ci/mmol) were obtained from Du Pont-New England Nuclear. Recombinant human  $^{125}$ I-MIP-1 $\alpha$  (2000 Ci/mmol) used for determination of affinity constants was from Amersham. Recombinant human RANTES, MIP-1 $\alpha$ , monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-4, eotaxin, IL-8, GRO- $\alpha$ , 78 amino acid epithelial  
20 cell-derived neutrophil activator (ENA-78), IFN- $\gamma$  inducible protein 10 (IP-10), platelet factor 4 (PF-4), neutrophil activating peptide 2 (NAP-2), stromal cell derived factor 1 $\alpha$  (SDF-1 $\alpha$ ) and lymphotactin, mouse eotaxin, and rat RANTES and MIP-1 $\alpha$  were purchased from PeproTech. Recombinant mouse MIP-1 $\alpha$  and human eotaxin, GRO- $\gamma$  and I309 were  
25 from R&D Systems. Recombinant human IFN- $\gamma$  was from Genzyme. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was purchased from Cascade Biochem Ltd. The following were generous gifts: guinea pig eotaxin from Dr. G. Andrews and Dr. H. J. Showell (Pfizer Central Research, Groton, CT, USA); human IL-5 from Dr. T. N. C. Wells (Glaxo-Wellcome Molecular, Geneva, Switzerland);  
30 and human C5a from Dr. J. Van Oostrum (Ciba Geigy, NJ, USA). Heparin

(6 kDa) and heparan sulphate from porcine or bovine intestinal mucosa, respectively, were purchased from Sigma. Rabbit antiserum to purified VV Lister 35 kDa protein, kindly provided by A. H. Patel (Institute of Virology, Glasgow, UK), or VV WR B15R protein expressed in baculovirus have  
5 been described (Patel *et al.*, 1990; Alcamí & Smith, 1992).

### Preparation of medium for binding and biological assays

Supernatants from orthopoxvirus-infected TK-143B cells or baculovirus-infected Sf cells were harvested at 1 or 3 days postinfection,  
10 respectively, and prepared as described (Alcamí & Smith, 1992). Baculovirus supernatants were concentrated and dialysed against PBS as described (Symons *et al.*, 1995). VV supernatants were inactivated with 4,5',8-trimethylpsoralen and UV light for cell migration assays (Tsung *et al.*, 1996).

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### Binding assays

Binding medium was RPMI 1640 containing 20 mM HEPES (pH 7.4) and 0.1% BSA. Cross-linking experiments with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) or ethylene glycolbis  
20 succinamidyl succinate (EGS) (1 mg/ml) to  $^{125}\text{I}$ -chemokines (0.4 nM) or  $^{125}\text{I}$ -IFN- $\gamma$  (2 nM) were performed in 25  $\mu\text{l}$  as described (Upton *et al.*, 1992; Alcamí & Smith, 1995b). Samples were analysed by SDS-PAGE in 16 or 20 cm long gels with 12 or 14% acrylamide. In the competition assays with U937 cells, supernatants were preincubated with 100 pM  $^{125}\text{I}$ -chemokine  
25 in 100  $\mu\text{l}$  for 1 h at 4°C. Subsequently,  $2.5 \times 10^6$  U937 cells were added in 50  $\mu\text{l}$  and incubated for 2 h at 4°C. Bound  $^{125}\text{I}$ -chemokine was determined by phthalate oil centrifugation as described (Alcamí & Smith, 1992). Scintillation proximity assay (Amersham) (Bosworth & Towers, 1989) was used for determination of the affinity constant. Purified 35K-Fc (200 pg)  
30 was incubated with  $^{125}\text{I}$ -MIP-1 $\alpha$ , with or without unlabelled chemokines, in

100  $\mu$ l for 2 h at room temperature. Protein A-fluorospheres containing scintillant were added in 50  $\mu$ l and incubated for 2 h, and bound  $^{125}$ I-MIP-1 $\alpha$  was determined by scintillation proximity assay in a beta counter. Non-specific binding, determined in the presence of 1000-fold excess unlabelled MIP-1 $\alpha$  or as binding to 200 pg of purified B8R-Fc, was subtracted and represented 3.8-7.5% of total cpm bound. Binding data were analysed by the Ligand program (Munson & Rodbard, 1980). The number of binding sites for MIP-1 $\alpha$  per molecule of vCKBP was calculated from the number of binding sites deduced from the Scatchard analysis and the concentration and molecular weight of recombinant 35K-Fc.

#### Construction of recombinant baculovirus

The 35 kDa gene was obtained by PCR using VV Lister DNA and oligonucleotides 5'-ATCGGTACCAATTATGAAACAATATATCGTCC-3' [SEQ ID NO: 1], which inserts a *Kpn*I site, and 5'-GTTGGATCCTCAGACACACGCTTTGAGTTTTG-3' [SEQ ID NO: 2], containing a *Bam*HI site. The 782-bp fragment was sequenced and cloned into pAcCL29-1 (Livingstone & Jones, 1989) generating pAc35K. The sequence contained His 148 instead of Arg 148 (Patel *et al.*, 1990), but both sequences are natural variants of the VV Lister 35 kDa protein and bind chemokines with the same specificity. Recombinant 35 kDa protein containing a C-terminal 6 histidine tag (35K.His) was produced in the baculovirus system. The 35 kDa gene was obtained by PCR using pCOS35K-Fc DNA (see 'Construction of 35K-Fc fusion and expression' below) and oligonucleotides 5'-TCAGAATTCATTATGAAACAATATATCGTCC-3' [SEQ ID NO: 3], containing an *Eco*RI site and 5'-ATCCTCGAGGACACACGCTTTGAGTTTTG-3' [SEQ ID NO: 4] containing an *Xho*I site. The 778bp fragment was cloned into pBAC-1(Novagen) generating pAc35K.His. The sequence was confirmed and contained the

Arg 148 variant of the Lister 35 kDa protein. The recombinant baculoviruses Ac35K and Ac35K.His were produced from pAc35K and pAc35K:His, respectively, as described (Alcamí & Smith, 1995b). The 35K.His protein was purified by metal chelate affinity chromatography (Ni-NTA resin; Qiagen Inc.). The recombinant baculovirus AcB15R and AcB8R have been described (Alcamí & Smith, 1992; Alcamí & Smith, 1995b).

### Construction of 35K-Fc fusion and expression

The VV Lister 35 kDa gene fused to the Fc region of human IgG1 was constructed in pCOSFCLINK (a generous gift of Dr P. R. Young, Smithkline Beecham Pharmaceuticals, USA). The insert was obtained by PCR using VV Lister DNA and the oligonucleotides 5'-TCAGAAATTCATTATGAAACAATATATCGTCC-3' [SEQ ID NO: 3], which introduces an *Eco*RI site, and 5'-ATCGGTACCGACACACGCTTTGAGTTTTG-3' [SEQ ID NO: 5], which inserts Gly and Thr via a *Kpn*I site after amino acid 258 of 35 kDa protein prior to the hinge region of human IgG1, which was provided by pCOSFCLINK, generating pCos35K-Fc. The sequence was confirmed and contained the Arg 148 variant of the Lister 35 kDa protein. The 35K-Fc fusion protein was purified from supernatants of CHO cells stably transfected with pCOS35K-Fc on a protein A-Sepharose column (Pharmacia). The B8R-Fc fusion protein will be described elsewhere (J. A. Symons and G. L. Smith, unpublished).

### Measurement of calcium mobilisation in human eosinophils and neutrophils

Changes in intracellular calcium flux were determined in purified human neutrophils and eosinophils as described previously (Jose *et al.*, 1994). Briefly, granulocytes were separated from mononuclear leukocytes, red blood cells and platelets using a combination of dextran

sedimentation and density centrifugation over Percoll-plasma gradients. For neutrophil experiments, granulocytes comprised greater than 95% neutrophils and the remaining cells were a mixture of eosinophils and mononuclear cells. For eosinophil experiments, blood was obtained from healthy atopic volunteers and eosinophils were purified (>98%) from the isolated granulocytes by immunomagnetic separation using anti-human CD16 microbeads (midimacs, Miltenyl Biotec) (Anwar *et al.*, 1993). Purified cells were loaded with Fura-2 AM (neutrophils 1  $\mu$ M, eosinophils 2.5  $\mu$ M) and, after two washes, resuspended at  $10^6$  cells/ml in  $\text{Ca}^{2+}$ /Mg $^{2+}$ -free PBS containing 10 mM HEPES, pH 7.4, 10 mM glucose and 0.25% BSA. Aliquots of cells were taken and the external calcium concentration adjusted to 1 mM. Changes in fluorescence were monitored at 37°C using a spectrometer (LS50, Perkin-Elmer Corp) at excitation wavelengths 340 and 380 nm, and emission wavelength 510 nm.

15

#### **Chemotaxis assay**

Cell migration was evaluated in 10 mm tissue culture inserts (Gibco BRL) placed in 24 well plates. A 0.5 ml aliquot of test reagents diluted in RPMI 1640 with 1 mg/ml BSA was placed in the lower compartment and 0.5 ml of U937 cell suspension ( $4 \times 10^6$  cells/ml) were placed in the upper chamber, separated by a polycarbonate filter (8  $\mu$ m pore size). After incubation at 37°C for 2 h the filter was washed, fixed, and stained with DAPI (Sanderson *et al.*, 1996). The number of migrating cells in five high powered fields were counted using a fluorescence microscope.

25

#### **Bioassay of eosinophil accumulation in guinea pig skin**

##### **i) Accumulation of endogenous peripheral blood eosinophils.**

Sedated guinea pigs were pretreated for 1 h with human IL-5 (18 pmol/kg; i.v. injection) to induce blood eosinophilia (Collins *et al.*, 1995). Guinea pig eotaxin, human C5a or LTB $_4$ , with or without purified

30

35K.His protein, were then injected intradermally (50 µl/site) into individual sites in the shaved dorsal skin of the animals. After 2 h the animals were sacrificed and the skin sites excised (11 mm diameter) for quantification of eosinophil accumulation by measurement of eosinophil peroxidase as described (Collins *et al.*, 1995).

#### ii) Accumulation of $^{111}\text{In}$ -labelled eosinophils.

Eosinophils were purified from the peritoneal cavity of horse serum-treated donor animals and radiolabelled with  $^{111}\text{InCl}_3$  as described (Jose *et al.*, 1994).  $^{111}\text{In}$ -eosinophils ( $5 \times 10^6$  cells/animal) were injected intravenously into recipient animals and after 10 min guinea pig eotaxin and human C5a, with or without purified 35K.His protein, were injected intradermally (100 µl/skin site; 2 sites/treatment) into individual sites in the shaved dorsal skin. After 2 h the animals were sacrificed and the skin sites excised (17 mm diameter) for quantification of eosinophil accumulation by measurement of  $^{111}\text{In}$  counts using a gamma counter (Jose *et al.*, 1994).

## Results

### VV, cowpox and camelpox viruses encode soluble chemokine binding activity

Although no CKR homologues were found in the VV genome sequence (Goebel *et al.*, 1990), we searched for the expression of soluble chemokine binding proteins by orthopoxviruses. Binding assays with  $^{125}\text{I}$ -RANTES were performed with media from cultures infected with 13 strains of VV, rabbitpox or buffalopox viruses (considered VV strains), cowpox virus (Brighton Red strain), elephantpox virus (considered a cowpox virus strain) or camelpox virus, followed by chemical cross-linking with EDC. A  $^{125}\text{I}$ -RANTES-vCKBP complex was observed with several viruses, including VV Evans (Fig. 1c), but not the best characterised VV strains Western Reserve (WR) and Copenhagen (Fig. 1a). The size of the ligand-



vCKBP complex was 33-37 kDa, suggesting a vCKBP size of 25-29 kDa after subtraction of the 8 kDa monomeric RANTES. The variation in vCKBP size may reflect different polypeptide lengths or degrees of glycosylation among viruses. The different size predicted for vCKBP from  
5 orthopoxviruses by Graham *et al.* (Graham *et al.*, 1997) in similar experiments (41 kDa) might be due to the use of higher resolution gels in this study. The soluble vCKBP was expressed before (early) and after (late) viral DNA synthesis by VV rabbitpox and USSR (Fig. 1b). The RANTES-vCKBP complex was 37-44 kDa when the cross-linker EGS was  
10 used (Fig. 1b), suggesting that two RANTES molecules could form a complex with vCKBP. Similar results were observed with  $^{125}\text{I}$ -MIP-1 $\alpha$ , another CC chemokine (Fig. 1c). MIP-1 $\alpha$  can dimerise but there is controversy about binding of CC chemokines as monomers or dimers to cellular CKRs (Murphy, 1996). Cross-linking of media from all viruses  
15 shown in Figure 1a to  $^{125}\text{I}$ -IL-8 (not shown) or  $^{125}\text{I}$ -GRO- $\alpha$  (Fig. 1c, not shown) was negative or very weak, suggesting vCKBP specificity for CC, but not CXC, chemokines.

#### **The VV Lister 35 kDa secreted protein encodes vCKBP**

20 Although the predicted vCKBP size was 25-29 kDa, its expression profile in orthopoxviruses (Fig. 1a,c) correlated with expression of a 35 kDa major secretory protein in VV Lister, Evans and rabbitpox but not in WR, Tian-Tan or Wyeth (Patel *et al.*, 1990; Mart'nez-Pomares *et al.*, 1995). The 35 kDa gene is present in cowpox (Pickup *et al.*, 1982), but is  
25 truncated by a frameshift mutation within the signal peptide in VV Copenhagen, where it is called B29R or C23L (Goebel *et al.*, 1990), and in VV WR, where it produces a 7.5 kDa protein (Venkatesan *et al.*, 1982; Patel *et al.*, 1990). The expression of vCKBP before (early) and after (late) viral DNA synthesis (Fig. 1b) was also consistent with the transcriptional

regulation of the 35 kDa gene from the strong early/late promoter p7.5, widely used in VV expression vectors (Smith, 1993).

The absence of RANTES-binding activity in supernatants from cultures infected with a VV Lister mutant lacking the 35 kDa gene (Lister $\Delta$ 35K) (Patel *et al.*, 1990), indicated that the 35 kDa protein is the vCKBP encoded by VV Lister (Fig. 2a). This was confirmed by showing specific binding of  $^{125}$ I-RANTES to the 35 kDa protein produced from recombinant baculovirus (Ac35K) and when fused to the Fc region of human IgG1 (35K-Fc), but not to the controls AcB15R (VV IL-1 $\beta$ R) (Alcami & Smith, 1992), AcB8R (VV IFN- $\gamma$ R) (Alcami & Smith, 1995b) or B8R-Fc (VV IFN- $\gamma$ R fused to human IgG1; J. A. Symons and G. L. Smith, unpublished) (Fig. 2a). Additional evidence was the neutralisation of the RANTES binding activity of VV Lister and Ac35K supernatants by antiserum against purified 35 kDa protein (Patel *et al.*, 1990) but not by antiserum against the VV IL-1 $\beta$ R B15R (Alcami & Smith, 1992) (Fig. 2b).

Recently, the myxoma virus soluble IFN- $\gamma$ R (Upton *et al.*, 1992), but not the VV IFN- $\gamma$ R (B8R) (Alcami & Smith, 1995b), was demonstrated to bind a wide range of chemokines by interaction with their proteoglycan-binding domain (Graham *et al.*, 1997; Lalani *et al.*, 1997). Here we demonstrate that VV expresses a 35 kDa vCKBP that does not bind IFN- $\gamma$  and is not B8R by showing that: i) the binding of  $^{125}$ I-RANTES to natural (VV Lister) or recombinant (Ac35K and 35K-Fc) 35 kDa protein was inhibited by unlabelled RANTES but not IFN- $\gamma$  (Fig. 2a); ii)  $^{125}$ I-IFN- $\gamma$  was cross-linked to the natural IFN- $\gamma$ R expressed from all three viruses WR, Lister and Lister $\Delta$ 35K, or the recombinant IFN- $\gamma$ R (AcB8R) but not to 35 kDa protein (Ac35K) (Fig. 2c); iii) the molecular size of monomeric IFN- $\gamma$  complexed to IFN- $\gamma$ R were 60 or 52 kDa for the natural or recombinant IFN- $\gamma$ R, respectively (Alcami & Smith, 1995b), which was higher than the 35 kDa size of the RANTES-vCKBP complex and could not be explained by the size difference between IFN- $\gamma$  (17 kDa) and RANTES (8 kDa) (Fig.

2a,c); iv) the 35 kDa size of the RANTES-vCKBP complex was smaller than the 50 kDa size predicted for the RANTES-IFN- $\gamma$ R complex, as reported for the myxoma IFN- $\gamma$ R/M7T (Lalani *et al.*, 1997); and v) the binding of  $^{125}$ I-IFN- $\gamma$  to the natural (VV Lister) or recombinant (AcB8R) VV IFN- $\gamma$ R was inhibited by unlabelled IFN- $\gamma$  but not RANTES (Fig. 2c). It is also demonstrated that VV IFN- $\gamma$ R does not bind RANTES by the inability of RANTES to bind recombinant IFN- $\gamma$ R (AcB8R and B8R-Fc) (Fig. 2a) and the lack of binding of RANTES to supernatants from cells infected with Lister $\Delta$ 35K, which still expresses IFN- $\gamma$ Rs (Fig. 2a,c). In addition, a higher molecular size of the soluble IFN- $\gamma$ R, known to be encoded by all the orthopoxviruses included in this study (Alcami & Smith, 1995b), complexed with  $^{125}$ I-RANTES,  $^{125}$ I-GRO- $\alpha$  or  $^{125}$ I-IL-8 was not observed (Fig. 1a,c, not shown), indicating that the IFN- $\gamma$ R from other strains of VV, cowpox or camelpox viruses does not bind CC or CXC chemokines.

15

#### **Binding specificity and affinity of vCKBP for chemokines**

The binding specificity of vCKBP was further investigated in cross-linking experiments (Fig. 3). vCKBP expressed from recombinant baculovirus, VV Lister, cowpox virus or camelpox virus bound human  $^{125}$ I-RANTES and this binding was effectively competed in a dose-dependent manner by all human CC chemokines tested, including eotaxin, a specific chemoattractant of eosinophils. In contrast, human CXC chemokines or the C chemokine lymphotactin were not bound by vCKBP. The failure of high doses of GRO- $\alpha$  or IL-8 to block  $^{125}$ I-RANTES binding was consistent with the negative or very weak cross-linking of vCKBP from orthopoxviruses to  $^{125}$ I-GRO- $\alpha$  or  $^{125}$ I-IL-8 (Fig. 1c, not shown), suggesting a very low, and probably physiologically insignificant, affinity for CXC chemokines. vCKBP also bound CC chemokines from mouse and rat. This vCKBP binding specificity was confirmed in experiments cross-linking  $^{125}$ I-MIP-1 $\alpha$  to VV Lister supernatants in the presence of unlabelled chemokines (not shown).

30

These results demonstrate that vCKBP from VV, cowpox and camelpox viruses bind CC, but not CXC, chemokines.

The affinity of vCKBP for CC chemokines was determined in binding studies of purified recombinant 35K-Fc to  $^{125}\text{I}$ -MIP-1 $\alpha$  and quantification of bound MIP-1 $\alpha$  by scintillation proximity assay (Bosworth & Towers, 1989). The interaction of MIP-1 $\alpha$  to 35K-Fc was saturable and of high affinity: in two experiments  $K_D$  values of  $103 \pm 4$  pM (Fig. 4a) and  $128 \pm 9$  pM (not shown) were obtained. The high affinity interaction of vCKBP for CC chemokines ( $K_D$  115 pM) was similar or 100-fold higher than the affinity of chemokines for cellular CKRs (Howard *et al.*, 1996; Murphy, 1996; Premack & Schall, 1996). The Scatchard analysis of the binding data predicted 4.2 binding sites for MIP-1 $\alpha$  in the 35K-Fc protein in both experiments, suggesting 2.1 binding sites for chemokines in the 35 kDa protein. This was consistent with binding of two chemokine molecules to vCKBP as suggested by cross-linking studies (Fig. 1c). The interaction of  $^{125}\text{I}$ -MIP-1 $\alpha$  with 16.5 pM 35K-Fc was inhibited by a similar dose of purified recombinant 35 kDa protein, produced in baculovirus and containing a C-terminal 6 histidine tag (35K.His), suggesting that both proteins bind MIP-1 $\alpha$  with similar affinities (Fig. 4b).

The affinity of vCKBP for other chemokines was determined in binding assays of 35K-Fc to  $^{125}\text{I}$ -MIP-1 $\alpha$  in the presence of increasing doses of unlabelled chemokine competitors (Fig. 4c). These data showed that vCKBP binds CC chemokines with different affinities: MIP-1 $\alpha$  > eotaxin > RANTES > MCP-1 > I309. The lower affinity for I309 probably reflects the amino acid sequence of this chemokine being the most divergent of the CC chemokine group (Murphy, 1996). Competition experiments with other chemokines corroborated, in a different quantitative binding assay, the specificity of vCKBP for CC, but not CXC or C, chemokines, and extended these studies to 19 chemokines (Fig. 4d).

**vCKBP does not bind to the proteoglycan binding site of chemokines**

Chemokines interact with glucosaminoglycans such as heparin or heparan sulphate through their carboxy terminus (Webb *et al.*, 1993; Witt & Lander, 1994). This interaction is thought to facilitate chemokine localisation to the endothelial cells and does not interfere with chemokines binding to their receptors. To ascertain whether vCKBP bound to the proteoglycan binding site of chemokines,  $^{125}\text{I}$ -MIP-1 $\alpha$  was preincubated with various doses of heparin or heparan sulphate before incubation with 35K-Fc protein. Figure 4e shows that heparin or heparan sulphate did not interfere with MIP-1 $\alpha$  binding to vCKBP at doses up to 10  $\mu\text{g/ml}$ , and had a minor effect at higher doses up to 200  $\mu\text{g/ml}$ , equivalent to a  $6 \times 10^5$ -fold molar excess of heparin over MIP-1 $\alpha$ .

**vCKBP blocks the binding of CC chemokines to cell surface receptors**

Biological activity of vCKBP for CC chemokines was shown by the ability of supernatants from orthopoxviruses and recombinant baculovirus Ac35K to inhibit the binding of radioiodinated RANTES and MIP-1 $\alpha$ , but not GRO- $\alpha$ , to cellular receptors (Fig. 5a). This again demonstrated the specificity of vCKBP for CC chemokines. As previously reported (Neote *et al.*, 1993; Wang *et al.*, 1993), excess unlabelled RANTES failed to block the binding of  $^{125}\text{I}$ -RANTES to cells (Fig. 5a). The binding of  $^{125}\text{I}$ -MIP-1 $\alpha$ , but not  $^{125}\text{I}$ -GRO- $\alpha$ , to U937 cells was inhibited in a dose-dependent manner by supernatants containing the 35 kDa protein (Fig. 5b). The potent inhibitory activity of vCKBP was illustrated by the complete blockade of MIP-1 $\alpha$  binding to cells in the presence of 0.1-1  $\mu\text{l}$  of medium, equivalent to the amount of vCKBP synthesised by only 200-2000 cells. Furthermore, 10 or 50 pM of purified recombinant 35K.His protein produced in baculovirus blocked the binding of 100 pM of  $^{125}\text{I}$ -MIP-1 $\alpha$  to U937 cells by 50% and 95%, respectively (Fig. 5c). These results showed that the soluble vCKBP blocks the binding of CC chemokines to their

cellular receptors and were consistent with the high affinity interaction of MIP-1 $\alpha$  and other CC chemokines with recombinant 35K-Fc.

**vCKBP blocks the *in vitro* biological activity of CC chemokines**

5           The blockade of CC chemokine binding to cell receptors suggested that vCKBP would inhibit their biological activity. This was demonstrated by the ability of vCKBP to inhibit the rapid and transient increase in intracellular calcium in eosinophils by the CC chemokines MCP-4 and eotaxin (Fig. 6a). This inhibitory effect was selective for these  
10 agonists since vCKBP had no effect on the calcium response induced by C5a in eosinophils (not shown). In contrast, vCKBP had no effect on the elevation of intracellular calcium levels in human neutrophils in response to the CXC chemokines IL-8, GRO- $\alpha$  and NAP-2 (Fig. 6b). This demonstrated specificity of vCKBP for CC, but not CXC, chemokines in a biological  
15 assay.

vCKBP from VV Lister or recombinant baculovirus Ac35K also blocked the migration of U937 cells *in vitro* in response to the CC chemokine MIP-1 $\alpha$  (Fig. 6c). In contrast, Lister $\Delta$ 35K or AcB8R supernatants did not.

20

**Blockade of eotaxin chemotactic activity by vCKBP *in vivo***

The blockade of chemokine activity by vCKBP was demonstrated *in vivo* in a guinea pig skin model of eotaxin-induced eosinophil infiltration (Collins *et al.*, 1995). Purified recombinant 35K.His  
25 bound guinea pig eotaxin, as determined by cross-linking assay (not shown). vCKBP inhibited local eosinophil infiltration induced by intradermal injection of eotaxin, but not other agents, in a guinea pig model using eosinophil peroxidase as an index of eosinophil numbers (Fig. 7a). The inhibitory effect of various doses of vCKBP on eosinophil accumulation in  
30 the skin in response to eotaxin was determined in guinea pigs injected

intravenously with  $^{111}\text{In}$ -eosinophils (Fig. 7b). The potency of this inhibitor *in vivo* was illustrated by the complete blockade of eotaxin activity by a 3-fold molar excess of vCKBP and a 50% inhibition by an equal molar concentration (Fig. 7a,b). This emphasises the potential therapeutic application of vCKBP in inflammatory diseases.

### Discussion

We have identified and characterised a soluble vCKBP from poxviruses. We demonstrate that vCKBP is the VV Lister 35 kDa secreted protein, and similar proteins are expressed by other orthopoxviruses. This protein is encoded by gene B29R or C23L in the VV strain Copenhagen (Goebel *et al.*, 1990), where it is truncated and inactive, and is transcribed from the strong, early-late promoter p7.5, frequently used in VV expression vectors (Smith, 1993).

In contrast to known cellular CKRs, which are hydrophobic proteins with seven transmembrane domains, the 35 kDa protein is a cysteine-rich, acidic (pI 4.2), soluble protein with no sequence similarity to cellular counterparts. Many of the poxvirus-encoded cytokine receptors mimic the extracellular binding domain of known cellular counterparts, thus vCKBP may represent a viral version of an unidentified cellular molecule, as has been proposed for the VV IFN- $\alpha/\beta$ R (Symons *et al.*, 1995).

Chemokine binding proteins closely related to the VV Lister 35 kDa protein are found in other VV strains, the leporipoxviruses Shope fibroma virus (S-T1) and myxoma virus (M-T1), racoonpox virus, cowpox virus and camelpox virus (Graham *et al.*, 1997) (this report), and are predicted to be produced by strains of variola virus, the cause of smallpox, (G3R) (Massung *et al.*, 1994; Shchelkunov, 1995). Interestingly, VV WR gene A41L is predicted to encode a secretory protein which is related, but more distantly so, to the VV Lister 35 kDa protein (Howard *et al.*, 1991;

Mart'nez-Pomares *et al.*, 1995) and may represent another vCKBP that binds chemokines not included in this study.

Concerning the specificity of vCKBP for different chemokines, we demonstrate unequivocally that it is a specific inhibitor of CC, but not CXC or C, chemokines. This is shown by cross-linking experiments with cold competitors, in more quantitative binding experiments and by determination of affinity constants, in studies in which 19 chemokines were analysed. Most importantly, the CC specificity was confirmed in biological assays showing that vCKBP does not block the interaction of CXC to cellular receptors nor their capacity to transduce signals. This contradicts the conclusion of Graham *et al.* (Graham *et al.*, 1997) who reported it had a broad specificity for CC and CXC chemokines. However, consistent with our results, these authors found that excess IL-8 could not compete the cross-linking of RANTES to vCKBP, but the effect on the biological activity of CXC chemokines was not investigated.

The mechanism by which vCKBP inhibits chemokine activity might be its interaction with the proteoglycan binding domain of chemokines, which is thought to facilitate chemokine localisation to endothelial cells, as suggested by Graham *et al.* (Graham *et al.*, 1997). Alternatively, vCKBP might interact with the receptor binding domain of chemokines and thus block binding to cellular receptors, as reported for poxvirus soluble receptors (Alcami & Smith, 1995a; Smith, 1996). The data in this paper show that vCKBP blocks CC chemokine activity by binding chemokines with high affinity, at a site different to the proteoglycan binding domain, and preventing their interaction with cellular receptors. Consistent with this, the affinity of vCKBP for human MIP-1 $\alpha$  (K<sub>D</sub> 115 pM) is similar or 10- to 100-fold higher than the affinity of most chemokines for cellular CKRs, which are normally in the range of 1-10 nM (Howard *et al.*, 1996; Murphy, 1996; Premack & Schall, 1996). vCKBP also binds eotaxin, RANTES and MCP-1 with high affinity (K<sub>D</sub> 1-15 nM). The lower affinity of



RANTES for myxoma M-T1 protein reported by Graham *et al.* (Graham *et al.*, 1997) ( $K_D$  73 nM) might be due to the use of human chemokines with the M-T1 protein from myxoma virus, whose natural host is the rabbit.

There is precedent for myxoma virus soluble cytokine receptors binding  
5 human cytokines with much lower affinity than rabbit cytokines (Mossman *et al.*, 1995; Schreiber *et al.*, 1996).

Evidence for the binding of vCKBP to two chemokine molecules was found in cross-linking experiments using EGS and in the Scatchard analysis of the binding of MIP-1 $\alpha$  to 35K-Fc. MIP-1 $\alpha$  and IL-8  
10 can dimerise but there is controversy about binding to cellular CKRs and biological activity of chemokines as monomers or dimers (Murphy, 1996). If vCKBP does bind to dimeric chemokines, this might enhance the inhibitory activity of vCKBP.

Poxviruses have evolved two different mechanisms to block  
15 chemokine activity. First, the soluble IFN- $\gamma$ R from myxoma virus (M-T7), but not VV strain WR, binds the heparin-binding domain of a wide range of chemokines (Graham *et al.*, 1997; Lalani *et al.*, 1997) and this additional function of M-T7 is consistent with an increased leukocyte infiltration to sites of myxoma virus replication in rabbits infected with a deletion mutant  
20 lacking this protein (Mossman *et al.*, 1996). The myxoma M-T7 protein may prevent the correct localisation of chemokines *in vivo*, which form a gradient by interacting with proteoglycans at the surface of endothelial cells. We show here that the soluble IFN- $\gamma$ R from 19 orthopoxviruses (Alcamí & Smith, 1995b), including strains of VV, cowpox and camelpox  
25 viruses, does not bind CC or CXC chemokines. Second, we show that orthopoxviruses encode a vCKBP which binds chemokines with high affinity, at a site different to the proteoglycan binding domain, and blocks their interaction with cellular CKRs.

The expression of a potent, soluble, CC-specific vCKBP by  
30 poxviruses suggests an important role for CC chemokines in anti-viral

defense. Nonetheless, deletion of the 35 kDa protein in VV rabbitpox did not greatly affect the outcome of infection in mice and rabbits (Mart'nez-Pomares *et al.*, 1995). Graham *et al.* (Graham *et al.*, 1997) now report an increased leukocyte influx in the skin of rabbits infected with VV rabbitpox mutant lacking the 35 kDa gene, compared to wild type VV rabbitpox. However, construction of a revertant virus in which the 35 kDa gene is re-introduced into the VV rabbitpox mutant genome is required to support this conclusion. This VV rabbitpox mutant was constructed by transfecting DNA from VV Lister into VV rabbitpox-infected cells (Mart'nez-Pomares *et al.*, 1995), and other mutations might have been introduced in the regions flanking the 35 kDa gene, which includes a gene encoding a TNFR homologue (Goebel *et al.*, 1990). Further studies with this or alternative models of poxvirus infection are required to fully understand the role of vCKBP in poxvirus pathogenesis.

The important role of chemokines and CKRs in the pathogenesis of virus infections is emphasised by the expression of i) chemokine-like proteins by molluscum contagiosum virus (Senkevich *et al.*, 1996), Kaposi's sarcoma-associated herpes virus (Moore *et al.*, 1996; Nicholas *et al.*, 1997) and murine cytomegalovirus (MacDonald *et al.*, 1997), representing potential alternative ways to modulate chemokine activity, and ii) seven-transmembrane-domain CKRs by large DNA viruses such as herpesviruses and poxviruses (Smith, 1996). Moreover, the MIP-1 $\alpha$  knockout mouse is less able to clear influenza virus infection (Cook *et al.*, 1995) and RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  increase resistance to HIV infection (D'Souza & Harden, 1996; Fauci, 1996).

Poxvirus proteins that counteract the immune system have been optimised during the evolution of viruses with their hosts and are providing insights into the physiological role of immune regulatory molecules such as cytokines (Alcami & Smith, 1996) or chemokines, and represent potential sources of immunomodulatory proteins and new

strategies of immune modulation. A novel soluble chemokine inhibitor from poxviruses with therapeutic potential is described herein. Eosinophil accumulation in response to eotaxin is a feature of allergic inflammatory reactions such as those occurring in allergic asthma. The potent eotaxin  
5 inhibitory effect of vCKBP *in vivo* emphasises its potential therapeutic application in inflammatory diseases. vCKBP has binding specificity for CC chemokines but the soluble inhibitor, or variants from other poxviruses, may provide the structural scaffolding needed to design specific soluble inhibitors of other chemokines.

10 The finding of a soluble vCKBP also has implications for infectious agents such as HIV in which CKRs play an important role in determining transmission and disease progression (D'Souza & Harden, 1996; Fauci, 1996). Inhibition of the interaction of the HIV envelope with the chemokine binding domain of CKRs has recently emerged as a new  
15 target for intervention. vCKBP might be engineered to create soluble molecules that bind to the domain of gp120 which interacts with the CKR in order to block HIV infection at an early stage (D'Souza & Harden, 1996; Fauci, 1996). A similar approach might yield therapeutic agents to block attachment of the malarial parasite to the Duffy antigen on erythrocytes and  
20 initiation of the infection (Horuk *et al.*, 1993).

The poxvirus chemokine inhibitor reported here represents a new and exciting virus immune evasion mechanism which provides insights into virus pathogenesis, the function of key immune regulatory molecules and new strategies for therapeutic intervention in immune responses and  
25 disease.

### **Example 2**

#### **Inhibition of macrophage-tropic HIV replication by a soluble CC chemokine binding protein (vCKBP) from VV**

## Material and Methods

### Reagents

Recombinant human  $^{125}\text{I}$ -RANTES (2000 Ci/mmol) was obtained from Amersham. Recombinant human RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1 $\alpha$  were purchased from R&D Systems. Sodium heparin was purchased from Rovi. Phytohaemagglutinin (PHA) was purchased from Gifco and recombinant IL-2 (Proleukin) from Cetus. Recombinant soluble CD4 was a kind gift of Dr. R. Sweet (SmithKline Beecham, PA).

### 10 Viruses and plasmids

The following HIV strains were used: BAL, and NL4.3 have been previously described (Adachi *et al.*, 1986; Gartner *et al.*, 1986; and were kindly provided by Dr. Y. Chen (UCLA, Los Angeles) and Dr. A Rabson (NIH, Bethesda). Concentrated viral particles were obtained by  
15 centrifugation at 400,000 *g* for 10 min using a Beckman TL100 ultracentrifuge.

### Cells and culture conditions

PBLs were isolated by Ficoll-Hypaque gradients from healthy  
20 donors and cultured in RPMI supplemented with 10% FBS (Bio Whitaker), L-glutamine and antibiotics. Macrophage-derived monocytes (MDM) were isolated by adherence of isolated PBLs in RPMI medium supplemented with 10% FBS and 10% human AB serum (Bio Whitaker) in 24 well plates ( $2 \times 10^6$  cells per well). The purity of macrophage cultures was assessed  
25 by flow cytometry using the CD14 antibody (Coulter) and by peroxidase staining. MT2 is a T cell line immortalised by HTLV-I. MT2-R5 was generated in our laboratory by transfection of the CCR5 cDNA gene cloned in the pCDNA3 plasmid (Invitrogen) under the control of the human cytomegalovirus immediate early promoter. Geneticin-resistant cells were  
30 selected after three months in culture and CCR5 expression was assessed

by flow-cytometry using the 5D7 antibody (Pharmingen). More than 65% of cells stained positive for CCR5. Cells were maintained in RPMI medium supplemented with 10% FBS. Stable HeLa cell transfectants (P4C5) expressing both the CD4 and CCR5 HIV co-receptors and the LacZ gene under the control of the HIV LTR (a kind gift from Dr. F. Arenzana, Institut Pasteur, Paris) were generated by transfection with expression vectors and selection in neomycin and puromycin. HeLa-ADA-gp120 cells (a kind gift from Dr. M. Allizon, Institut Cochin, Paris) express constitutively the gp160 from the ADA strain of HIV and harbour a retroviral vector expressing the HIV genes tat and rev. These HeLa clones were maintained in DMEM supplemented with 10% FBS.

Macrophage viability was assessed by measuring the incorporation of  $^{14}\text{C}$ -leucine. Macrophages were cultured for 12 h in leucine-free medium before adding 1  $\mu\text{Ci}$  of  $^{14}\text{C}$ -leucine. After 12 h cells were washed, scrapped and proteins were precipitated with TCA.  $^{14}\text{C}$ -leucine incorporation was measured in a beta counter (Wallac).

#### HIV infection assays

PHA-activated PBLs were infected with 5 ng of the Bal strain of HIV in the presence of vCKBP or control supernatants from baculovirus culture (AcNPV). After viral adsorption for 12 h, cells were extensively washed with PBS, seeded in 24 well plates and maintained in RPMI supplemented with 20% FBS and 20 ng/ml of recombinant IL-2. Twice a week, one half of the medium was removed and replaced by fresh medium. Viral replication was assessed by measuring p24 HIV antigen in culture supernatants.

MT2 and P4C5 cells were infected with Bal strain of HIV at a dose of 200 pg/ml and 20 ng/ml of p24 respectively.

At 6 to 7 days after their isolation, macrophages were infected with BAL (range 50 pg-200 ng/ml). In all the cell systems used,

vCKBP (an amount of supernatant equivalent to  $6 \times 10^5$  cell/ml) or control supernatants from baculovirus were added simultaneously during viral adsorption for 12 h. After infection, cells were extensively washed with PBS and fresh medium was added together with vCKBP and different  
5 controls. In some experiments viral preparations were previously incubated with vCKBP for 2 h at room temperature before infection. Viral replication was quantified by assessing p24 production in culture supernatants at different times after infection.

When chemokines or heparin were added to macrophage  
10 cultures, infection conditions were modified as follows: cells were washed twice with PBS before infection and fresh medium was added to cultures. The dose of HIV BAL strain was 50 pg of p24 and viral adsorption was performed during 2 h. Chemokines were added simultaneously at a concentration of 100 ng/ml and heparin was used at 200 µg/ml. After viral  
15 adsorption, cells were washed 3 times with PBS and the same dose of chemokines were added to cultures immediately after infection and at day 4 after infection. Culture supernatants were collected at days 4 and 7 and p24 levels were measured.

## 20 Fusion Assays

Co-cultures of P4C5 and ADA-gp120 HeLa cells were performed in 96 well plates ( $2 \times 10^4$  cells/well) as previously described (Oberlin *et al.*, 1996) and inhibition of fusion by 35K was assessed measuring beta-galactosidase levels.

25

## Recombinant 35K protein

Recombinant baculoviruses expressing the VV strain WR IL- $1\beta$  receptor (AcB15R) or the 35 kDa chemokine binding protein from VV strain Lister (Ac35K) have been described (Alcami & Smith, 1992; Alcami  
30 *et al.*, 1998). Supernatants from baculovirus-infected Sf cell cultures were

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30 *et al.*, 1998). Supernatants from baculovirus-infected Sf cell cultures were

concentrated and Dialysed against PBS as described (Alcami *et al.*, 1998).  
The VV 35 kDa or B8R recombinant proteins fused to human  
immunoglobulin (Fc35K and FcB8R, respectively) have been described  
elsewhere (Alcami *et al.*, 1998) (Symons and Smith, unpublished reagent).

5

### Binding assays

Binding of  $^{125}\text{I}$ -RANTES to Fc35K was quantified by  
scintillation proximity assay (Amersham) as described (Alcami *et al.*, 1998).  
Briefly,  $^{125}\text{I}$ -RANTES and Fc fusion protein were incubated for 2 h at room  
10 temperature. Protein A-fluorospheres containing scintillant were  
added and incubated for 2 h, and bound  $^{125}\text{I}$ -RANTES was determined in  
a beta counter. Crosslinking experiments with EDC or EGS were  
performed as described (Alcami *et al.*, 1998).

15 

## Results

### vCKBP inhibits HIV replication in human monocytes

To investigate if VV vCKBP could influence HIV infection,  
MDMs were prepared from peripheral blood mononuclear cells (PBMC)  
and infected with the BAL strain of HIV-1 (a monocyctotropic variant) for 5 h  
20 at doses ranging from 50 to 200 pg of p24 antigen. Infection was  
performed with either HIV-1 alone or together with a 1:50 dilution of the  
supernatant from insect cells that had been infected with Ac35K  
(expressing vCKBP) or a control wild type baculovirus not expressing this  
protein (AcNPV). After infection, cultures were washed extensively with  
25 PBS and background levels of p24 antigen were determined in the last  
wash medium using an ELISA assay (Du Pont). vCKBP or control  
supernatants were added at days 1, 3 and 6 in order to maintain the  
potential neutralisation effect during incubation. HIV replication was  
assessed by measuring p24 antigen levels in the culture supernatant at 3,  
30 7 and 10 days postinfection.



Figure 9 shows that HIV infection in these cells (control) produced steadily increasing levels of p24 antigen that reached more than 20,000 pg/ml by 10 days postinfection. Similar levels of virus replication was observed in the presence of supernatants from wild type baculovirus (AcNPV). In contrast, HIV infection was severely impaired in the presence of vCKBP supernatants (Ac35K) and produced only 57 pg/ml of p24 by day 10. This demonstrates that vCKBP is blocking HIV replication.

To determine the mechanism by which HIV infection is inhibited, the experiment shown in Fig. 9 was repeated but with addition of vCKBP at different times. First, HIV was pre-incubated with vCKBP for 1 h at room temperature prior to addition to susceptible cells. Figure 10a shows that this treatment inhibited HIV replication to a similar degree to that observed in Fig. 9. Likewise, addition of vCKBP only during adsorption, inhibited HIV infection (Fig. 10b). Significantly, treatment with vCKBP after infection (Fig. 10c) was also able to block HIV replication in culture, although a burst of p24 production was observed early after infection. In the conditions tested, only a small percentage of cells are infected at early times and virus released subsequently from these cells is then able to infect other cells.

In summary, these results demonstrate the antiviral effect of vCKBP in HIV infection of macrophages. In addition, these results strongly suggest that vCKBP neutralises HIV virions by blocking an early step in the infection.

## **Mechanisms for inhibition of HIV replication**

The inhibition of HIV replication in human monocytes could be by a direct inhibitory effect of vCKBP on HIV due to an interaction with the viral envelope, or an indirect effect due to inhibition by vCKBP of a chemokine(s) able to increase HIV replication as illustrated in Figs. 11 and

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The first hypothesis is based on the fact that vCKBP interacts with several CC chemokines that are also bound by CKRs that act as HIV co-receptors. It is possible that vCKBP is able to interact with both CC chemokines and the HIV gp120 envelope (Fig. 11).

- 5           The second hypothesis, envisages vCKBP binding chemokine(s) that are required for HIV replication in macrophages, hence resulting in inhibition of HIV replication in this system (Fig. 12).

To examine which of these mechanisms was correct experiments were performed to i) look for a direct interaction between the  
10 HIV envelope and vCKBP, ii) analyse the anti-HIV effect of vCKBP in other cell systems, and iii) identify chemokines produced by human macrophages that could be involved in an enhancement of HIV replication in these cells.

#### 15   **Interaction between the HIV envelope and vCKBP**

Binding studies were performed to look for an interaction between the HIV envelope and vCKBP. The ability of HIV virions to inhibit the binding of  $^{125}\text{I}$ -RANTES to purified recombinant vCKBP fused to the Fc portion of human immunoglobulin (Fc35K) was determined in a  
20 scintillation proximity assay (Alcami *et al.*, 1998). Figure 13 shows that binding of RANTES to Fc35K was partially inhibited in the presence of purified HIV viral particles, but this was non-specific since supernatants from uninfected PBLs gave a similar level of inhibition. This was probably due to the presence of contaminant chemokines in the concentrated viral  
25 preparations used in the experiment. The addition of soluble CD4, which increase the affinity of gp120 for CKRs, had no effect.

As an alternative method to identify an interaction between HIV envelope and vCKBP, crosslinking assays were performed with recombinant vCKBP produced in the baculovirus system and  $^{125}\text{I}$ -  
30 RANTES (Alcami *et al.*, 1998). If vCKBP bound to the HIV envelope, the

addition of HIV might compete with the binding of vCKBP to  $^{125}\text{I}$ -RANTES. However, the addition of purified preparations of the CCR5-tropic BAL strain of HIV, in the absence or presence of soluble CD4, did not reduce binding or  $^{125}\text{I}$ -RANTES and vCKBP (data not shown). Consistent with  
5 this, vCKBP did not inhibit fusion of HeLa cells expressing the gp120 of a CCR5-tropic HIV strain with HeLa cells expressing CD4 at the cell surface, in a fusion assay performed as described (Oberlin *et al.*, 1996) (data not shown).

#### 10 **Anti-viral effect of vCKBP in other cell systems**

To determine if vCKBP could inhibit HIV replication in other cell types, vCKBP was added to HeLa cells expressing CD4 and CCR5 (P4C5 cells). As shown in Fig. 14, no differences in HIV replication were observed in these cells in the presence or absence of vCKBP. Addition of  
15 different concentrations of soluble CD4 did not change this result. Similarly, vCKBP did not inhibit replication of the BAL strain of HIV in MT2/CCR5 cells, a lymphocytic cell line stably transfected with CCR5 (data not shown). If vCKBP was binding to the envelope of BAL strain of HIV and this was how virus replication was inhibited in monocytes, vCKBP  
20 would have been expected to inhibit HIV BAL replication in other cells too. This was not the case.

The replication of HIV in PBLs was not inhibited in the presence of recombinant vCKBP. In fact, in some experiments a slight increase of the level of HIV replication was observed (data not shown).  
25 This effect can be due to competition of vCKBP with chemokines such as RANTES that strongly inhibit HIV entry in CD4 T lymphocytes.

#### **Different chemokines enhance HIV replication in macrophages**

As a protein-protein interaction between vCKBP and HIV  
30 gp160 was not identified, it was possible that the inhibition of HIV

replication was due to an indirect effect through inhibition of chemokine(s) that are present in the medium of cultured macrophages and that are able to increase HIV replication (Fig. 12). To test this hypothesis, macrophage cultures were washed before infection and viral adsorption was performed in the presence of different chemokines or with a mixture (cocktail) of RANTES, MCP-1 and MIP-1 $\beta$  (at the same concentrations). In addition, because chemokines bind heparin, viral replication was also assessed in the presence of heparin. As shown in Fig. 15, in the conditions tested, MCP-1, and MIP-1 $\beta$  enhanced HIV replication greatly, whereas RANTES induced a more modest increase in p24 antigen production and MIP-1 $\alpha$  had no effect. Interestingly, heparin decreased HIV replication in macrophage cultures and this effect was not due to cell toxicity as protein synthesis was not significantly modified by heparin. In fact, better viability was seen in heparin-treated macrophages as assessed by incorporation of  $^{14}\text{C}$ -leucine (Fig. 17). Infection of PBLs with the same heparin concentrations did not result in differences in HIV replication (data not shown).

### Discussion

Data presented here show that vCKBP inhibits dramatically HIV infection/replication in human macrophages. This could have been due to either a direct interaction between vCKBP and the HIV envelope, or the binding of CC chemokines that are required for HIV replication by vCKBP.

The first hypothesis is based on the fact that vCKBP interacts with different CC chemokines of the same type that are bound by CKRs that function as co-receptors for macrophage-tropic strains of HIV. Therefore, it was possible that vCKBP can bind to CC chemokines and the HIV envelope. So far, we have failed to demonstrate any direct interaction between vCKBP and the HIV envelope despite using different technical approaches: *in vitro* crosslinking, scintillation proximity assays and fusion

systems. The HIV envelope could not compete the binding of vCKBP to labelled RANTES, nor could vCKBP prevent fusion of cells expressing CD4 and CCR5 with cells expressing HIV gp120. In scintillation proximity assays some inhibition of RANTES binding to vCKBP was observed, but  
5 this effect was not specific for the HIV envelope as supernatants from uninfected PBMC gave a similar level of inhibition. This effect is probably due to the presence of contaminant chemokines in the highly concentrated viral preparations used in these experiments. The fact that NL4.3. (a CXCR4 tropic virus) also induces this mild inhibition argues against an  
10 specific effect of BAL particles.

While no direct evidence of vCKBP-HIV envelope has been obtained, these negative data do not rule out absolutely that a direct neutralization of HIV by vCKBP occurs. In particular, it is necessary to investigate the ability of recombinant gp160 from CCR5 strains to bind  
15 vCKBP. It is still possible that, due to its ability to bind chemokines, vCKBP might be engineered to create soluble molecules that bind to the domain of gp120 which interacts with cellular CKRs, and to block HIV infection at an early stage.

The anti-viral effect of vCKBP has been investigated in 3  
20 different cell systems: PBLs, HeLa cells stably transfected with both the CD4 and CCR5 (clone P4C5), and MT2/R5 cells, a CD4 cell line expressing the CCR5 receptor. The first system is relevant as CD4 T lymphocytes are the main target of HIV although it represents a complex model as mitogen-activated T cells secrete high levels of CC chemokines.  
25 The other two models are highly specific and provided a direct evaluation of HIV replication in two different cell environments lacking other factors that can introduce bias in the test.

No effect of vCKBP was observed in these 3 models.

Altogether, these data argue against a specific interaction between vCKBP

and HIV gp160, and suggest an indirect inhibition mechanism. The inhibition of HIV replication was restricted to human macrophages.

As we could not demonstrate a protein-protein interaction between vCKBP and HIV gp160 the other possibility was that inhibition of HIV replication was due to an indirect effect through inhibition of chemokine(s) that are able to increase HIV replication and that are present in the medium of cultured macrophages (Fig. 12). Data presented suggest that CC chemokines (MIP-1 $\beta$ , MCP-1 and RANTES) increase HIV replication in human macrophages and that this effect can be blocked by non-specific chemokine ligands such as heparin. Thus it seems likely that vCKBP blocks HIV infection by binding a chemokine critical for HIV replication in macrophages. Supernatants from cultured macrophages contain detectable levels of chemokines and HIV infection of macrophages increases the production of chemokines (Verani *et al.*, 1997). In addition, it has been reported that RANTES can increase HIV replication in macrophages (Schmidtayerova *et al.*, 1996) although contradictory results have been published (Moriuchi *et al.*, 1996). Recently, it has been shown that IL-10 increases CCR5 expression in human macrophages and enhances HIV infection (Sozzani *et al.*, 1998). Therefore, HIV infection of macrophages is the result of a complex balance in which the expression of CKRs, the blockade of such receptors by soluble chemokines, and positive transduction signals elicited by chemokines and cytokines, influence HIV entry and replication.

Our results demonstrate that vCKBP inhibits HIV replication in macrophages and this effect is restricted to this cell type, probably due to interference with a chemokine critical for HIV replication in the macrophage environment. Although, vCKBP did not modify significantly HIV replication levels in other cell types (mainly CD4 lymphocytes), blocking HIV infection and propagation in macrophages would be very important for the control of AIDS. Indeed, a broad spectrum of non-lymphoid cells of the macrophage

lineage (Dendritic cells, Langerhans cells) are the main targets in acute HIV infection (Zaitseva *et al.*, 1997). These first cells to be infected are critical in spreading HIV infection through viral production, migration to lymphoid organs and contact with CD4 T lymphocytes. In fact, only CCR5-  
5 trpoic HIV strains are found in early steps of HIV infection (Zhu *et al.*, 1993; van't Wout *et al.*, 1994) and this is probably due to the fact that these macrophagic cells do not express the CXCR4 co-receptor but only CC-chemokines receptors like CCR5 which is the main co-receptor of monocytotropic HIV strains (Zaitseva *et al.*, 1997). In this context, vCKBP  
10 could be useful in blocking both infection of primay target cells and HIV replication in this environment, thus arresting viral propagation and infection of CD4 T lymphocytes.

New drug regimens including protease inhibitors are able to decrease viral load to undetectable levels in plasma but proviral DNA  
15 persists in both peripheral blood and lymph nodes (Finzi *et al.*, 1997; Stellbrink *et al.*, 1997). Furthermore, in lymphoid organs HIV replicates at low levels as detected by *in situ* hybridization techniques (Cavert *et al.*, 1997; Tamalet *et al.*, 1997). Altogether, these results indicate that the efficacy of these drug regimens to eradicate HIV-infected cells is  
20 incomplete (Wong *et al.*, 1997). It seems then reasonable to look for alternative approaches to eliminate the viral reservoirs responsible for persistence and spread of the infection. In particular, infected macrophages probably represent a major hidden reservoir in AIDS-treated patient and a main source of viral spread and generation of mutants  
25 resistant to the pharmacological pressure of antiretroviral drugs (Fauci, 1996). Molecules like vCKBP could help to control residual virus by inhibiting HIV replication in macrophage reservoirs.



### Figure Legends

FIG. 1. Soluble chemokine binding activity produced by orthopoxviruses. *a*, Media from cultures uninfected (mock) or infected with the indicated orthopoxviruses were incubated with  $^{125}\text{I}$ -RANTES and  
5 treated with the cross-linker EDC. *b*, Media from infected cultures were either harvested 24 h postinfection (L) or treated with the inhibitor of DNA synthesis cytosine arabinoside (40  $\mu\text{g}/\text{ml}$ ) and harvested 7 h postinfection (E). Media from cultures uninfected (mock) or infected were incubated with  $^{125}\text{I}$ -RANTES and cross-linked with EDC. *c*, Media from cultures  
10 uninfected (mock) or infected were incubated with the CC chemokines  $^{125}\text{I}$ -RANTES or  $^{125}\text{I}$ -MIP-1 $\alpha$ , or the CXC chemokine  $^{125}\text{I}$ -GRO- $\alpha$  and treated with EDC or EGS. The amount of medium used was equivalent to  $2\text{--}7 \times 10^4$  cells. An autoradiograph of the SDS-PAGE analysis, with molecular masses in kDa, is shown. The positions of RANTES (R), MIP-1 $\alpha$   
15 (M), GRO- $\alpha$  (G) and ligand-receptor complexes (square brackets) are indicated.

FIG. 2. Identification of the VV Lister 35 kDa protein as the vCKBP. *a*, Cross-linking of  $^{125}\text{I}$ -RANTES with EDC to supernatants from cultures infected with the indicated VVs, recombinant baculoviruses or 20 ng of purified Fc fusion proteins, in the absence or in the presence of 1000-fold excess of RANTES (RAN) or IFN- $\gamma$ . *b*, Cross-linking of  $^{125}\text{I}$ -RANTES with EDC to supernatants from cells infected with VV Lister or Ac35K in the presence of 5  $\mu\text{l}$  of preimmune, anti-35K or anti-B15R rabbit serum. *c*, Cross-linking of  $^{125}\text{I}$ -IFN- $\gamma$  with EDC to supernatants from cultures infected  
25 with the indicated viruses. The amount of supernatant used was equivalent to  $2 \times 10^4$  cells (VV) or  $4 \times 10^3$  cells (baculovirus). An autoradiograph of the SDS-PAGE analysis, with molecular masses in kDa, is shown. The position of RANTES (R), IFN- $\gamma$  monomers (M), IFN- $\gamma$  dimers (D), and ligand-receptor complexes (triangles) are indicated.

FIG. 3. Binding specificity of vCKBP encoded by VV, cowpox and camelpox viruses. Cross-linking of 0.4 nM human  $^{125}\text{I}$ -RANTES with EDC to medium from infected cultures, in the absence (NC) or in the presence of increasing concentrations of unlabelled chemokines from 5 different species. The amount of medium was equivalent to  $1 \times 10^4$  cells for orthopoxviruses and  $2 \times 10^3$  cells for Ac35K. The concentrations of unlabelled chemokines were 50-, 100-, 500- or 2000-fold excess for VV Lister and Ac35K, and 100- or 2000-fold excess for cowpox and camelpox. An autoradiograph of the SDS-PAGE analysis showing the ligand-receptor 10 complexes is shown. Lptn (lymphotactin).

FIG. 4. Affinity constant of vCKBP binding to chemokines. a, Saturation curve and Scatchard analysis of  $^{125}\text{I}$ -MIP-1 $\alpha$  binding to 35K-Fc. Purified 35K-Fc protein was incubated with the indicated doses of  $^{125}\text{I}$ -MIP-1 $\alpha$  for 2 h and the radioactivity bound determined by scintillation 15 proximity assay with protein A-fluoromicrospheres containing scintillant. Mean ( $\pm$ SEM) specific binding of triplicate samples is shown. b, Competitive inhibition with purified 35K.His. Purified 35K-Fc protein was incubated in triplicates with 50 pM  $^{125}\text{I}$ -MIP-1 $\alpha$  in the presence of increasing doses of purified 35K.His produced in the baculovirus system. 20 The percentage of specific binding (mean  $\pm$ SEM) refers to the binding in the absence of competitor, which was 4235 cpm. c, Competitive inhibition with various doses of CC chemokines. Purified 35K-Fc protein was incubated in triplicates with 50 pM  $^{125}\text{I}$ -MIP-1 $\alpha$  in the presence of increasing doses of unlabelled human CC chemokines. The percentage of 25 specific binding (mean  $\pm$ SEM) refers to the binding in the absence of competitor, which was 4049 or 4561 cpm. The  $K_D$  values calculated from the data are indicated. d, Competitive inhibition with CC, CXC and C chemokines. Purified 35K-Fc protein was incubated in triplicates with 50 pM  $^{125}\text{I}$ -MIP-1 $\alpha$  in the presence of a 10000-fold excess of unlabelled 30 human (h), mouse (m) or rat (r) chemokines. The percentage of specific

binding (mean  $\pm$ SEM) refers to the binding in the absence of competitor, which was 4340 cpm. e, MIP-1 $\alpha$  binding in the presence of glycosaminoglycans. Increasing doses of heparin or heparan sulphate were incubated in triplicates with 50 pM  $^{125}$ I-MIP-1 $\alpha$  for 30 min before the  
5 addition of purified 35K-Fc protein. The percentage of specific binding (mean  $\pm$ SEM) refers to the binding in the absence of competitor, which was 3014 cpm.

FIG. 5. Inhibition of chemokine binding activity by vCKBP. a, Binding of 100 pM of  $^{125}$ I-RANTES,  $^{125}$ I-MIP-1 $\alpha$  or  $^{125}$ I-GRO- $\alpha$  to U937  
10 cells in the presence of supernatants from  $5 \times 10^4$  infected cells or 100-fold excess of unlabelled chemokine. Mean ( $\pm$ SEM) from duplicate samples is expressed as the percentage of counts binding in the presence of competitor compared to that without competitor, which was 6601, 10620 and 2351 cpm for RANTES, MIP-1 $\alpha$  and GRO- $\alpha$ , respectively. b, Binding  
15 of  $^{125}$ I-MIP-1 $\alpha$  or  $^{125}$ I-GRO- $\alpha$  to U937 cells in the presence of different amounts of culture supernatants or 100-fold excess of unlabelled MIP-1 $\alpha$  or GRO- $\alpha$ . Mean ( $\pm$ SEM) from duplicate samples and total cpm bound to cells as in a. c, Binding of  $^{125}$ I-MIP-1 $\alpha$  to U937 cells in the presence of different amounts of purified 35K.His. Mean ( $\pm$ SEM) from duplicate  
20 samples as in a and total radioactivity bound 4518 cpm.

FIG. 6. Blockade of chemokine biological activity by vCKBP *in vitro*. a, Elevation of intracellular calcium levels in human eosinophils by CC chemokines. MCP-4 or eotaxin were preincubated with the indicated doses of purified 35K.His or control supernatant for 5 min before their  
25 addition to human eosinophils and measurement of calcium mobilisation. The maximum elevation of intracellular calcium (mean  $\pm$ SEM) in three experiments with eosinophils from different donors is shown. Representative traces from one experiment are represented in the insets. b, Elevation of intracellular calcium levels in human neutrophils by CXC  
30 chemokines. IL-8, GRO- $\alpha$  or NAP-2 were preincubated with purified

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35K.His or control supernatant for 5 min before their addition to human neutrophils and measurement of calcium mobilisation. The maximum elevation of intracellular calcium (mean  $\pm$ SEM) in four experiments with neutrophils from different donors is shown. c, Chemotactic activity of MIP-1 $\alpha$  on U937 cells. The mean ( $\pm$ SEM) number of migrated cells per field under high power magnification (n=5) in the presence of MIP-1 $\alpha$  (50 ng/ml), with or without competitor, or  $10^{-7}$  M fMLP (line) is shown.

FIG. 7. Inhibition of eotaxin-induced eosinophil accumulation in guinea pig skin by vCKBP. a, Guinea pigs were pretreated for 1 h with human IL-5 to induce blood eosinophilia. Guinea pig eotaxin, human C5a or LTB<sub>4</sub> were injected intradermally in the presence of purified 35K.His (30 pmol/skin site) or control medium and eosinophil accumulation determined after 2 h. Results represent the number of eosinophils per skin site  $\pm$ SEM from n=4-5 animals. A statistically significant difference between groups that did or did not receive purified vCKBP is indicated by \* ( $p < 0.02$ ). The detection limit of the assay is indicated with a dashed line. No eosinophil accumulation was detected in response to the injection vehicle (Hanks' buffered salt solution containing 0.1% low endotoxin BSA), purified 35K.His or control medium alone. b, <sup>111</sup>In-eosinophils were injected intravenously into guinea pigs and after 10 min guinea pig eotaxin or human C5a, with or without 35K.His, were injected intradermally into individual sites. Accumulation of <sup>111</sup>In-eosinophils was determined after 2 h. Results represent mean  $\pm$ SEM from 4 animals. Eosinophil accumulation in response to the injection vehicle is indicated with a dashed line, and was similar to that obtained with purified 35K.His or control medium alone.

FIG. 8. Amino acid alignment of proteins. The amino acids sequences of VV strains WR (Smith *et al.*, 1991) and Copenhagen (Goebel *et al.*, 1990) A41L proteins, the comparable proteins from variola virus strains Harvey (Aguado *et al.*, 1992) and India 1967 (Shchelkunov *et al.*, 1994), the 35 kDa protein from VV strain Lister (Patel *et al.*, 1990), cowpox

virus (Hu *et al.*, 1994), and variola virus strain India 1967 (Shchelkunov *et al.*, 1994), and the Shope fibroma virus T1 protein (Upton *et al.*, 1987) are shown.

FIG. 9. Inhibition of HIV replication in macrophages by vCKBP. Human MDMs were cultured for one week and infected with BAL strain of HIV at 50 ng/ml of p24 alone (control) or in the presence of a 1/50 dilution of a baculovirus supernatant containing vCKBP (Ac35K) or a baculovirus control supernatant (AcNPV). Viral adsorption was performed during 5 h. Ac35K or AcNPV were added again at days 1, 3 and 6. Viral replication after infection (day 0) was assessed by p24 production in culture supernatants. Note that a logarithmic scale is used.

FIG. 10. Inhibition of HIV infection of macrophages by vCKBP. Human MDMs were cultured for one week and infected with BAL strain of HIV at 200 ng/ml of p24 alone (control) or in the presence of a 1/50 dilution of a baculovirus supernatant containing vCKBP (Ac35K) or a baculovirus control supernatant (AcNPV). Viral adsorption was for 5 h. Viral replication after infection (day 0) was assessed by p24 production in culture supernatants. Note that a logarithmic scale is used. (a). Infectious supernatants were preincubated with Ac35K or AvNPV 1 h at room temperature before infection of macrophages (neutralisation). No Ac35K or AcNPV supernatants were added thereafter. (b). Ac35K or AcNPV supernatants were added only during viral adsorption. (c). Ac35K or AcNPV were added after viral adsorption at days 1, 3 and 6.

FIG. 11. Model for inhibition of HIV replication by binding of vCKBP to the HIV envelope.

FIG. 12. Model for inhibition of HIV replication by binding of vCKBP to CC chemokines needed for HIV replication.

FIG. 13. Scintillation proximity assay. Purified Fc35K of FcB8R was incubated in duplicate with 130 pM  $^{125}$ I-RANTES in the presence of 1000-fold excess of unlabelled RANTES or 1  $\mu$ g of HIV virions

(BAL, NL4,3), in the presence or absence of 10 µg of soluble CD4, for 2 h. UCspnPBLs refers to preparation from non-infected PBL supernatants processed in the same conditions. The radioactivity bound was determined by scintillation proximity assay with protein A-fluoromicrospheres  
5 containing scintillant. The percentage of total binding refers to binding in the absence of competitor.

FIG. 14. vCKBP does not block HIV replication in stably transfected HeLa/CD4/CCR5 cells. The clone P4C5 that expresses both HIV co-receptors (CD4 and CCR5) was infected with 20 ng of p24 of the  
10 HIV BAL strain in the presence or absence of different concentrations of soluble CD4 (range 0.1-10 µg/ml). vCKBP from recombinant baculovirus ( $6 \times 10^5$  cells equivalent/ml) or control supernatants from wild type infected Sf cells were added during infection and maintained during the whole experiment. P24 antigen production was assessed at different times after  
15 infection. Levels of p24 at day 5 are shown. vCKBP alone or in the presence of different concentrations of soluble CD4 did not modify HIV replication.

FIG. 15. HIV replication in the presence of chemokines. Macrophages were isolated by adherence in 24 well plates and cultured in  
20 RPMI medium supplemented with 10%FBS and 10% AB serum. After 6 days in culture, cells were washed twice with PBS and fresh medium was added together with 50 pg of the HIV BAL strain and the indicated chemokines at a dose of 100 ng/ml. After 2 h of adsorption, cells were washed 3 times and fresh medium was added together with 100 ng/ml of  
25 the different chemokines or heparin. Viral replication was measured at days 4 and 7 post infection by quantifying p24 antigen production in culture supernatants. Values of 250 means levels were greater than 250 pg/ml and further dilutions are necessary to obtain exact values. Levels of p24 antigen are shown at the top of the bars.

## CLAIMS

1. A method for inhibiting infection of a target cell by a virus,  
5 which virus normally infects the target cell by binding to a chemokine receptor on the target cell, which method comprises contacting the target cell and solution containing the virus with an effective amount of an anti-viral agent which is a protein from the 35kDa major secretory protein family or a biologically active fragment thereof.
- 10 2. The method according to claim 1, wherein the anti-viral agent inhibits infection of the target cell by the virus by binding to a chemokine required by the virus for infection.
3. The method according to claim 2, for inhibiting infection of target cells by HIV.
- 15 4. The method according to any one of claims 1 to 3, for treatment or prophylaxis.
5. The use of a protein from the 35kDa major secretory protein family or a biologically active fragment thereof or a nucleic acid encoding the protein or fragment, together with a pharmaceutically acceptable  
20 carrier, in the manufacture of a medicament for treatment or prophylaxis of a viral infection.
6. The use according to claim 5, wherein the medicament is for the treatment or prevention of HIV infection in a subject.
7. The use according to claim 6, for the treatment or prevention  
25 of infection by CCR5, CCR4, CCR3 or CCR2 tropic HIV.
8. A medicament, comprising a recombinant poxvirus derived from a poxvirus which naturally encodes a chemokine-binding protein which is a member of the poxvirus 35 kDa major secretory protein family, the recombinant poxvirus genetically engineered to be incapable of  
30 expressing the functional chemokine-binding protein.



1/17

Fig. 1.

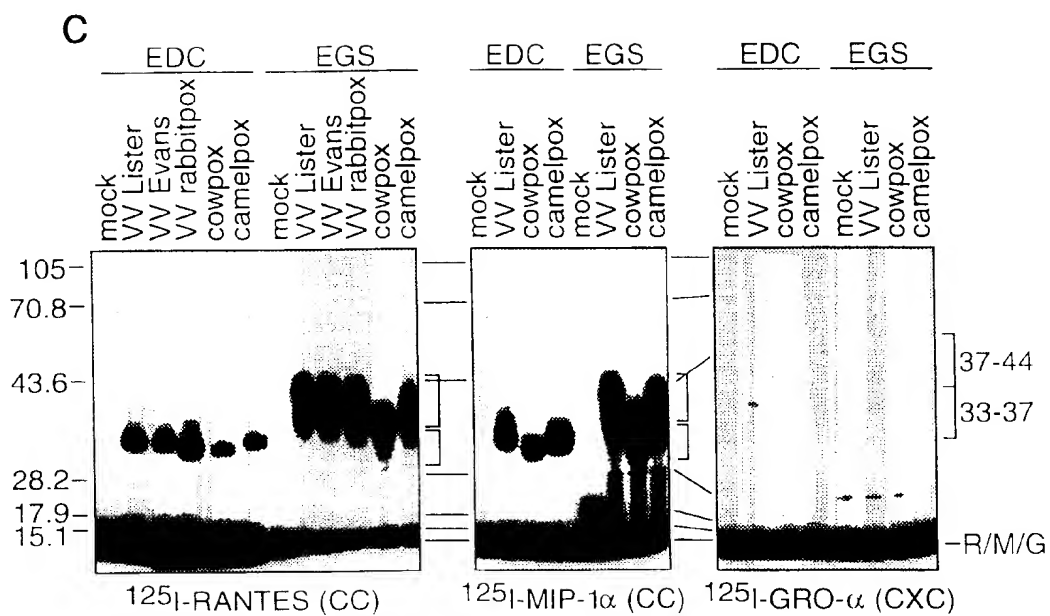
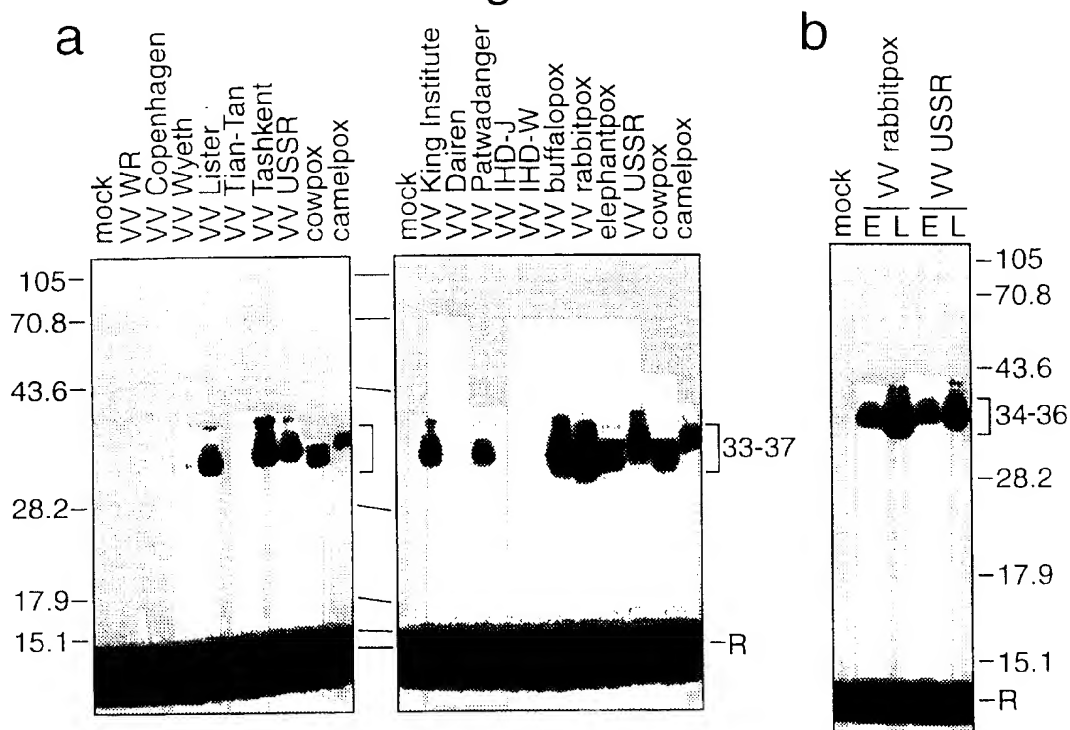
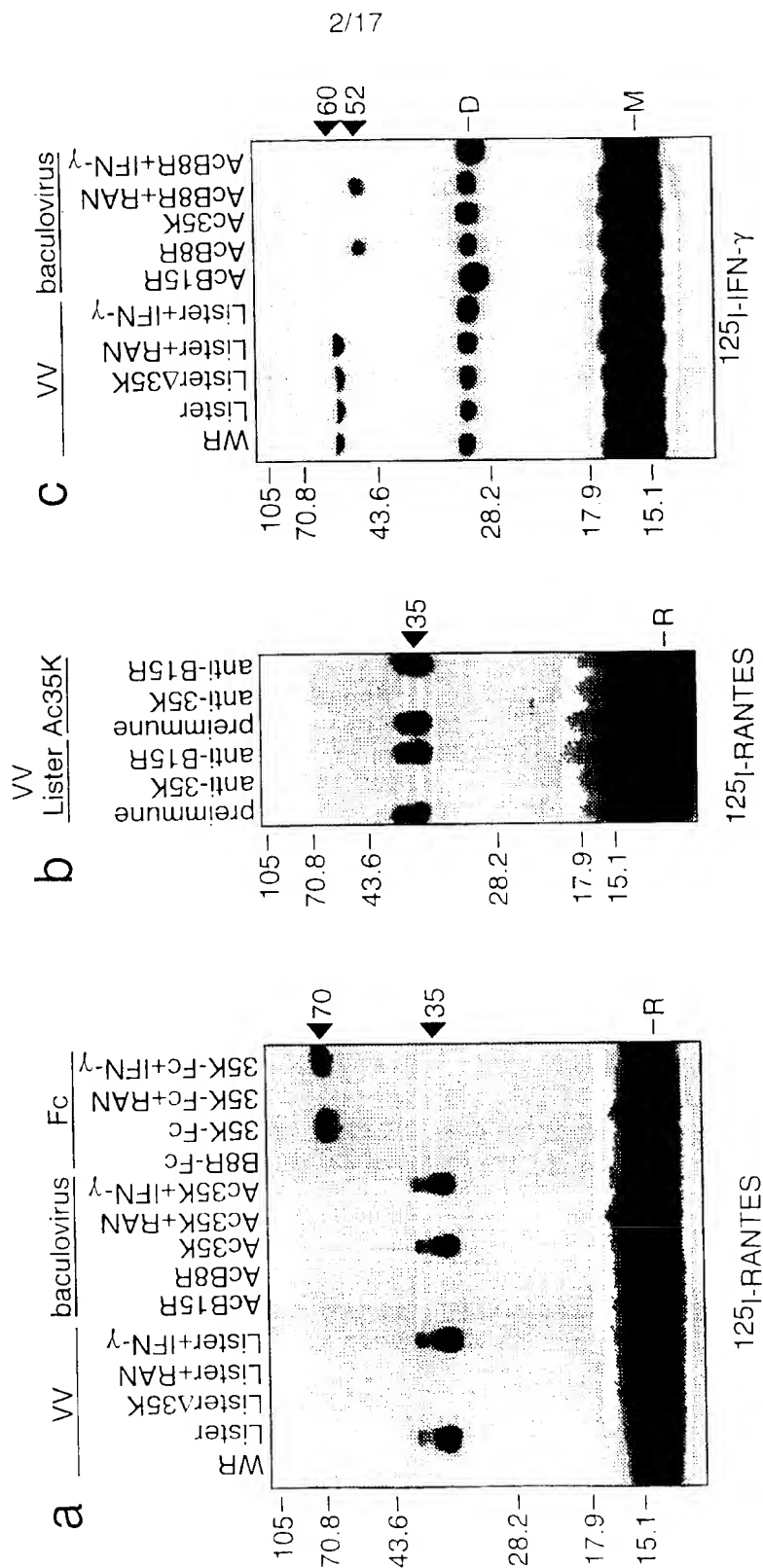
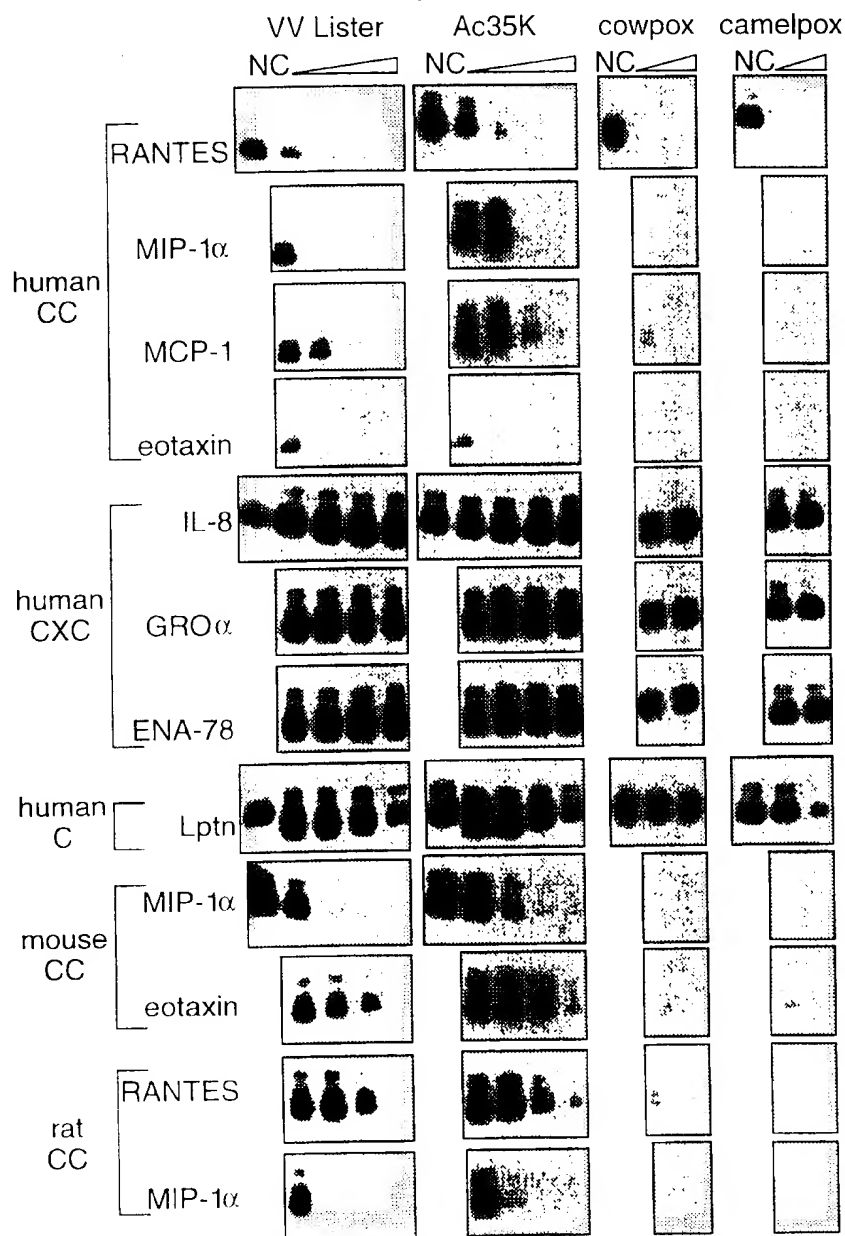


Fig.2.



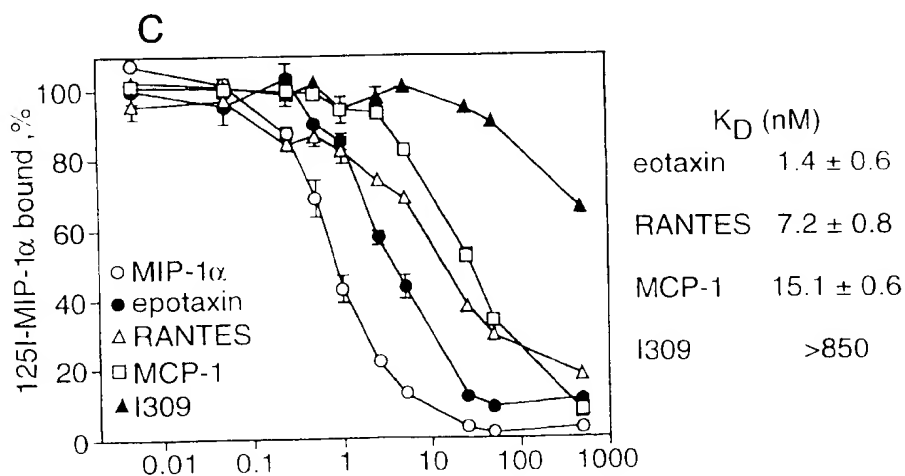
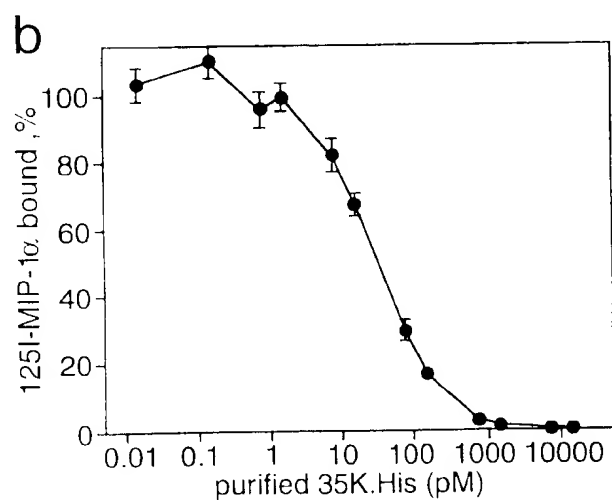
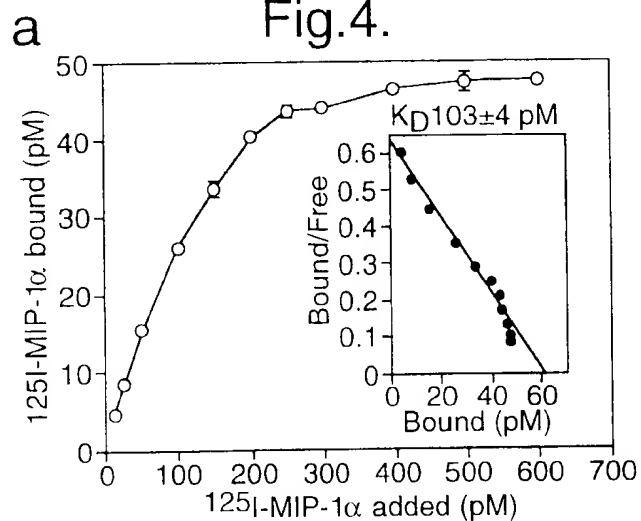
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Fig.3.



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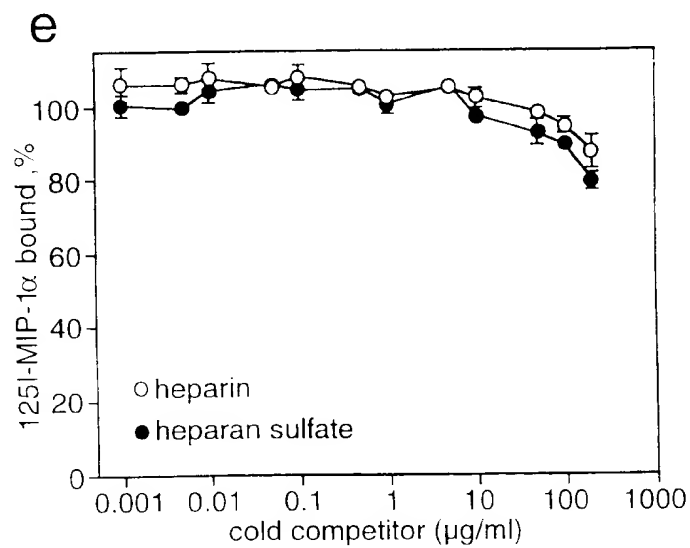
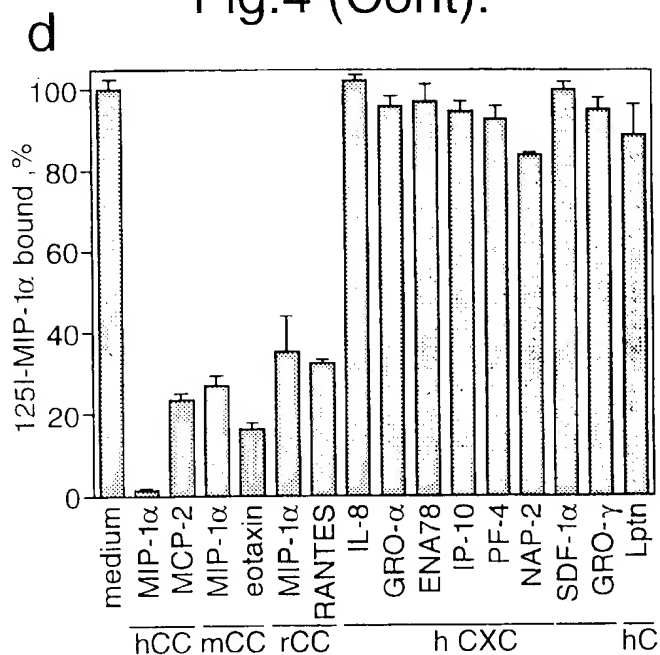
Fig.4.



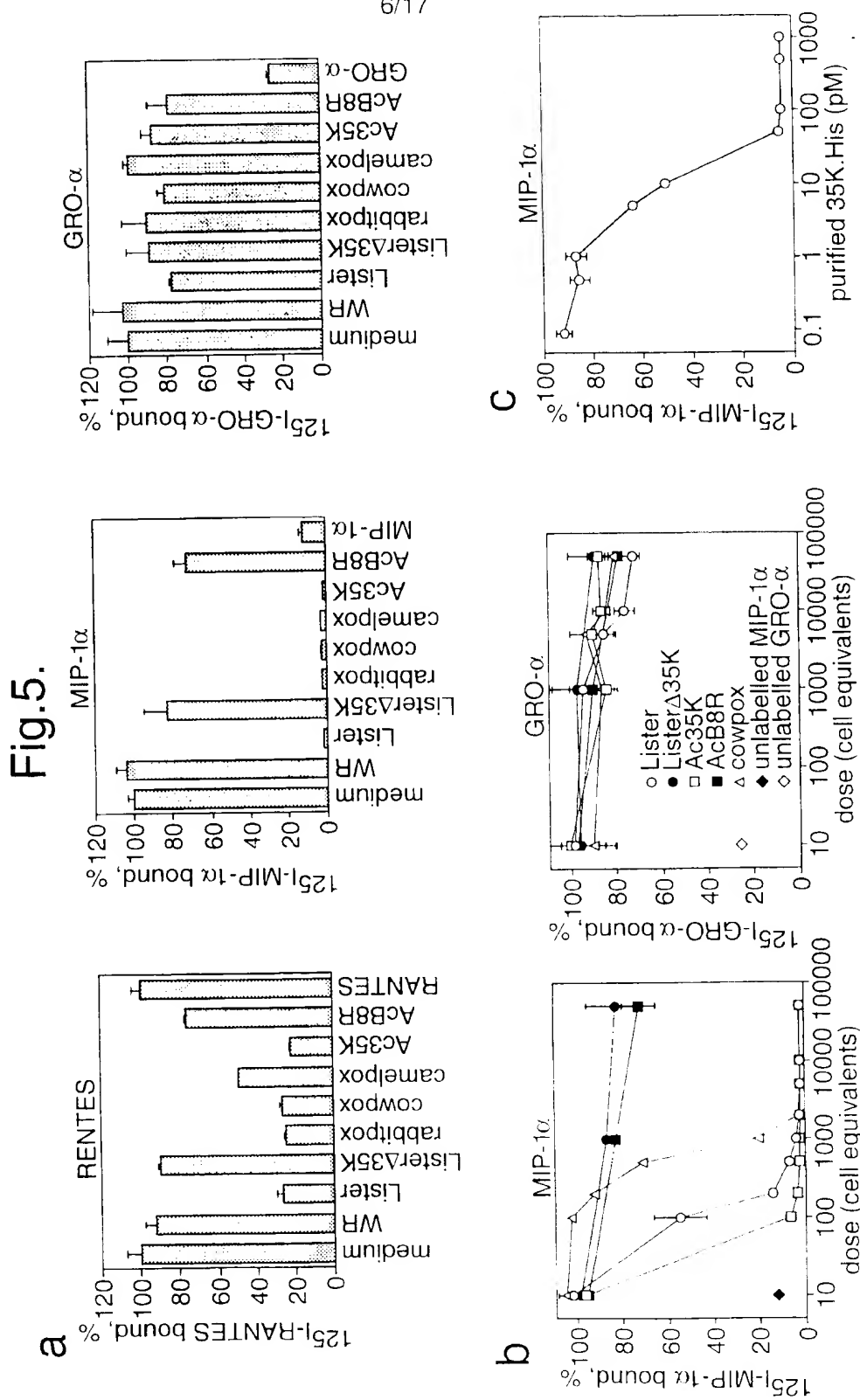
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Fig.4 (Cont).

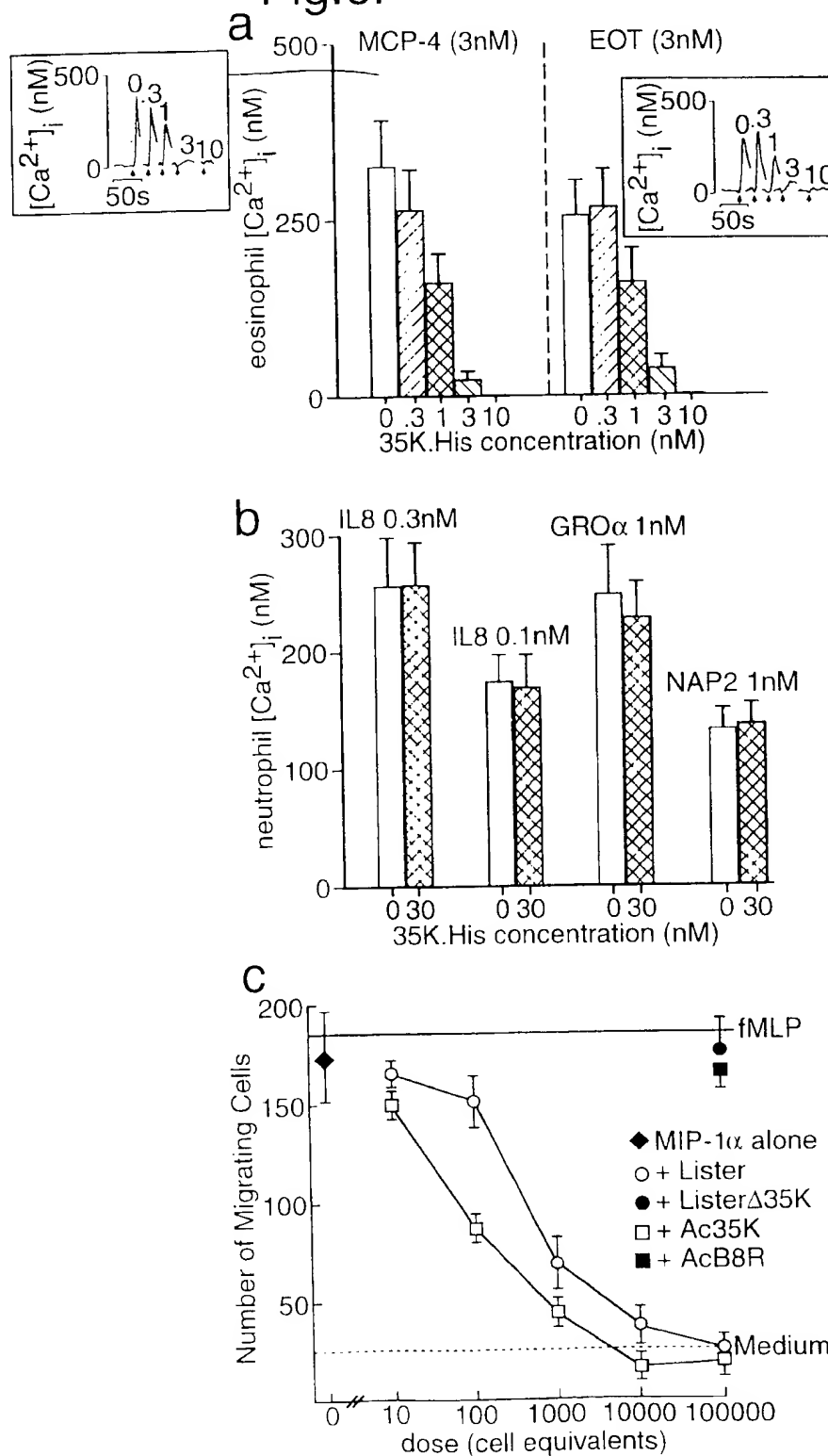


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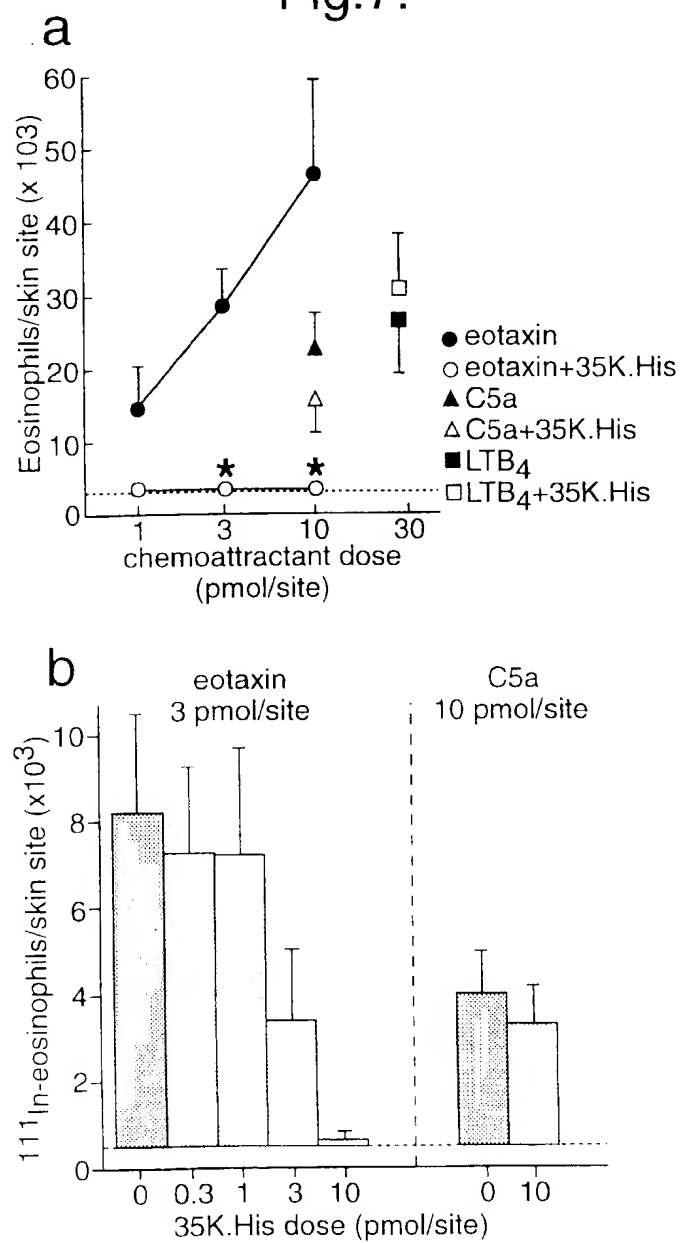
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Fig.6.

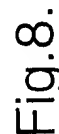


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Fig.7.







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Fig.9.

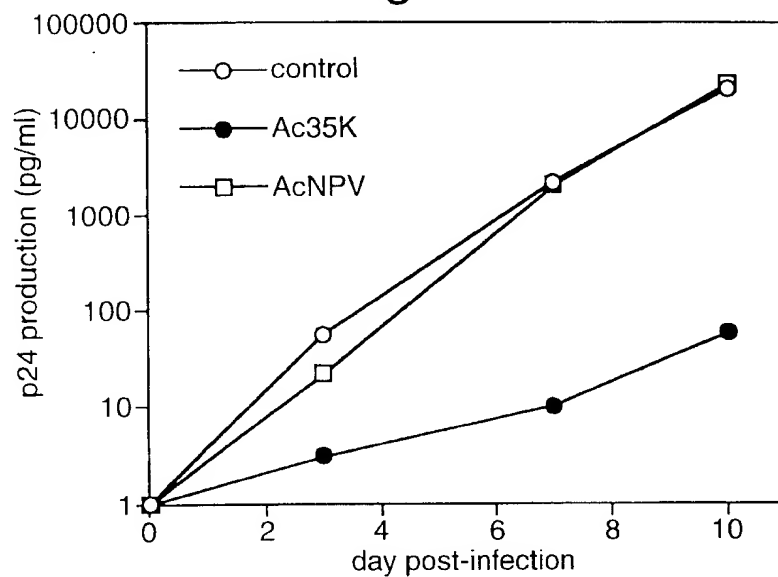
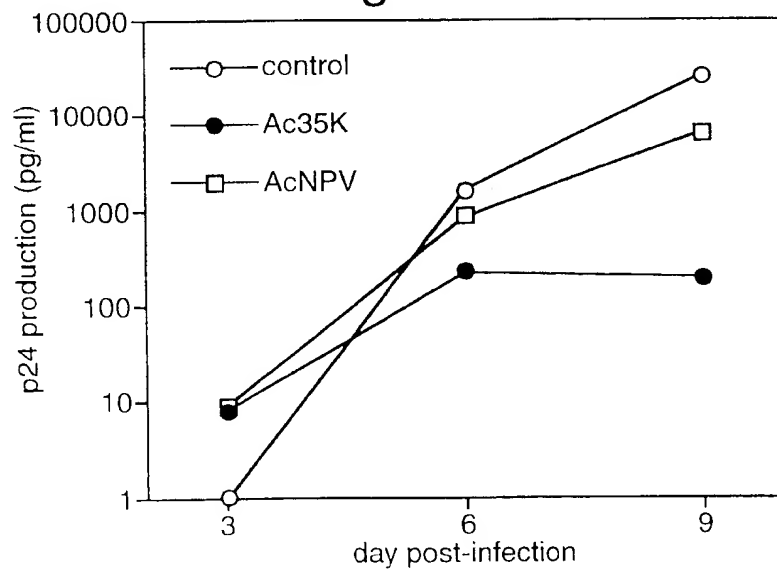
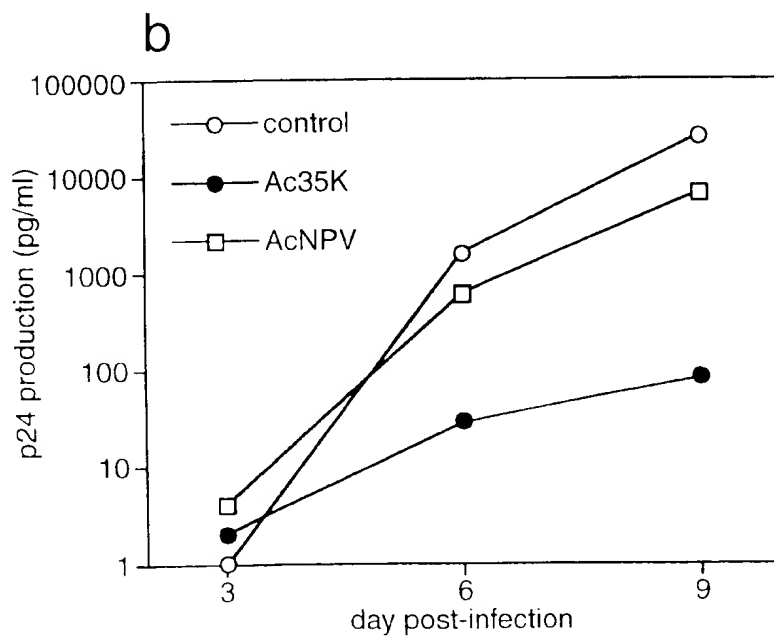
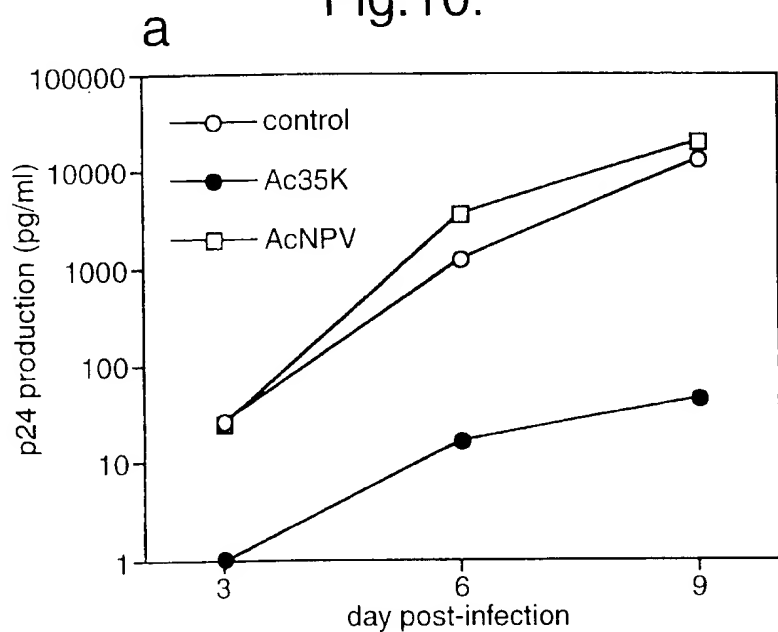


Fig.10c.



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Fig.10.



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Fig.11.

Ac35k directly interacts with  
HIV envelope blocking infection

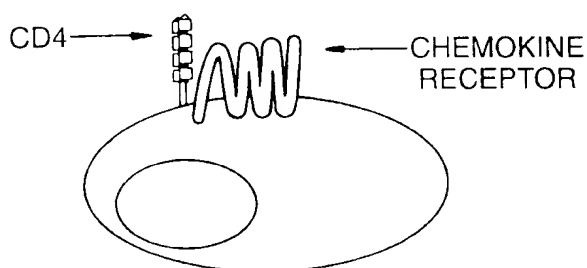
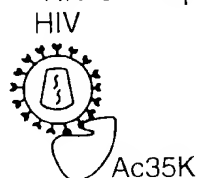
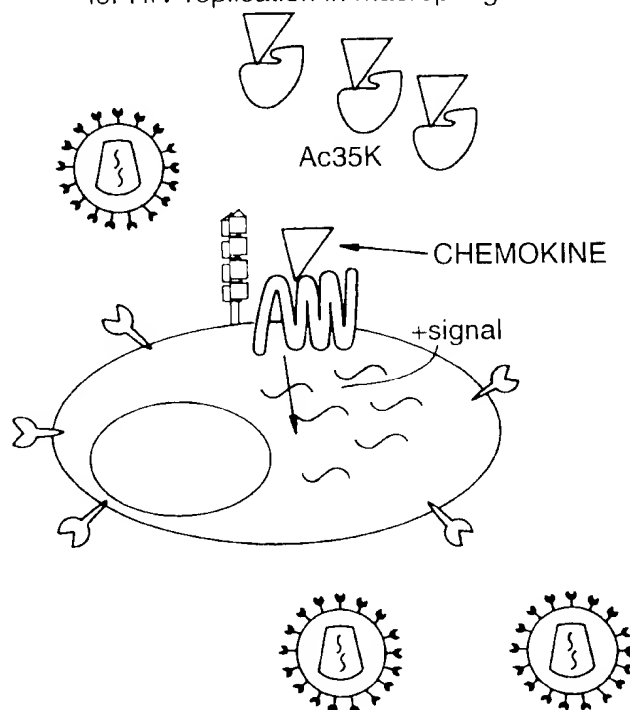


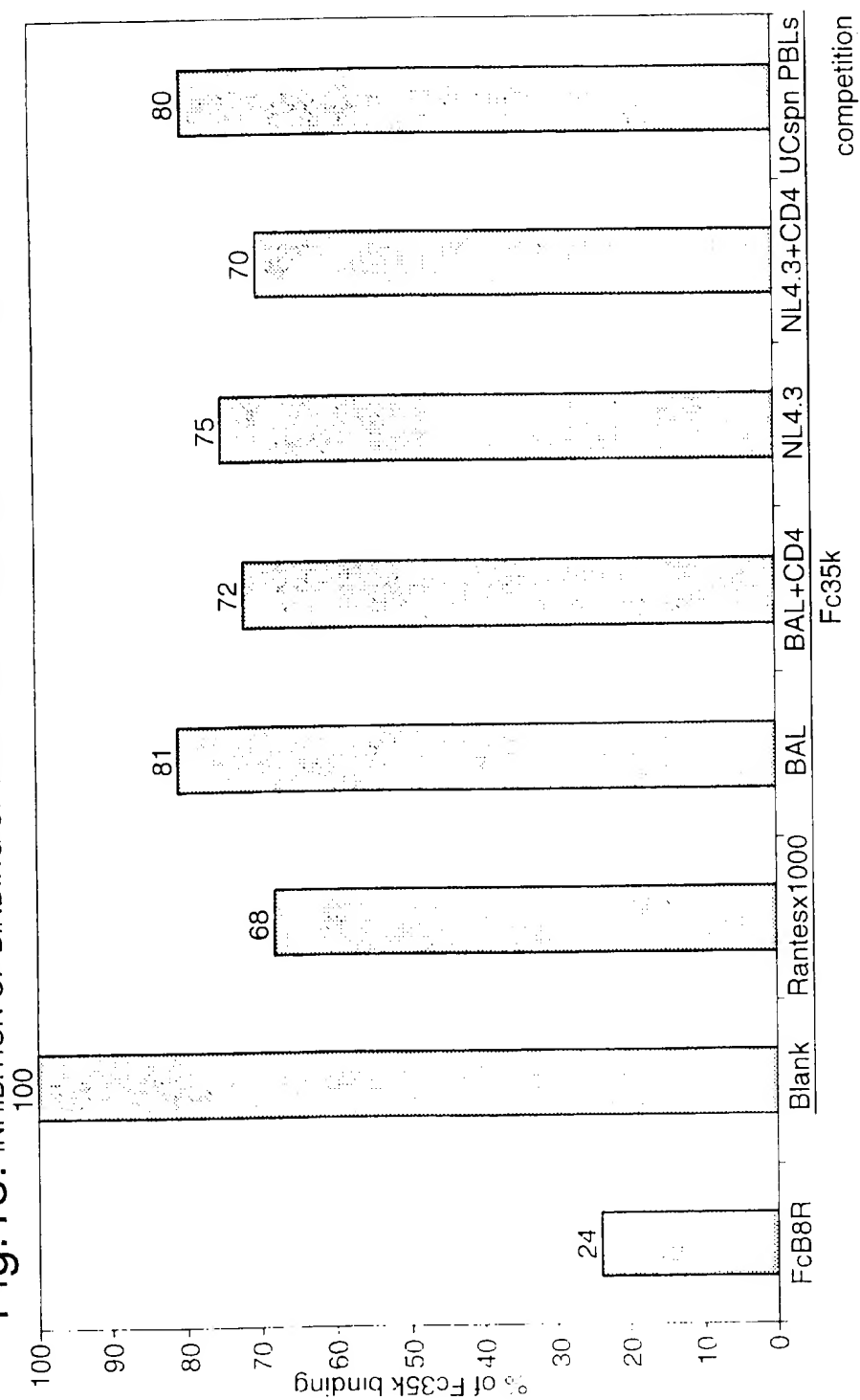
Fig.12.

Ac35k directly interacts with a chemokine required  
for HIV replication in macrophages



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Fig.13. INHIBITION OF BINDING OF RANTES TO Fc35K COUPLED BEADS



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Fig.14.

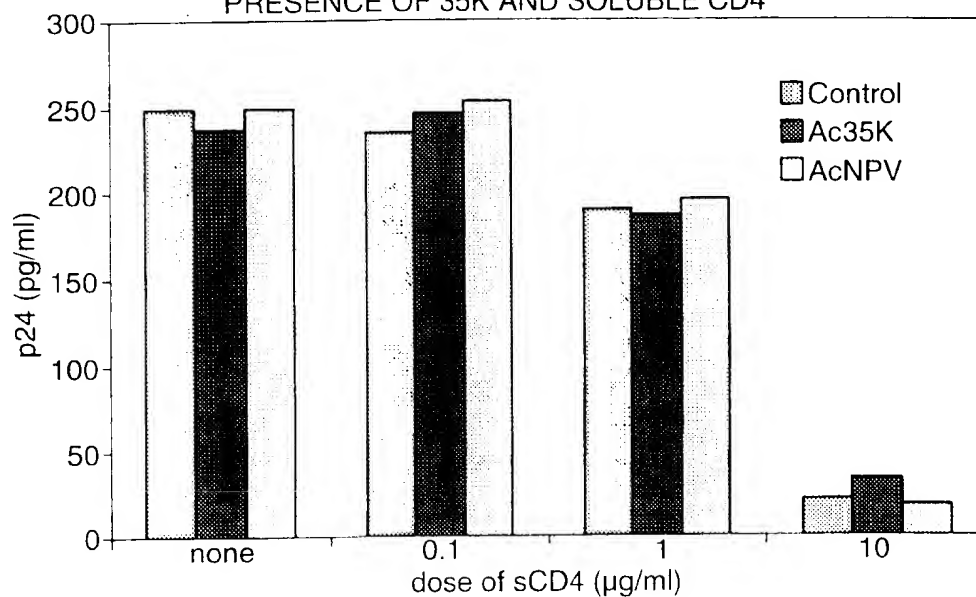
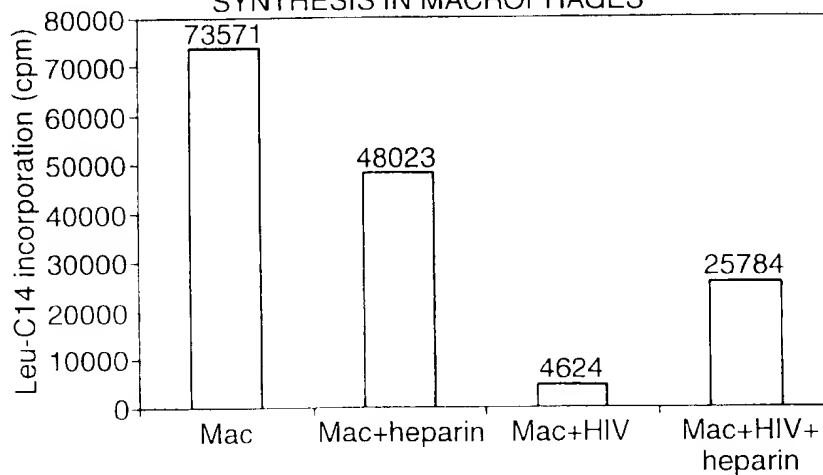
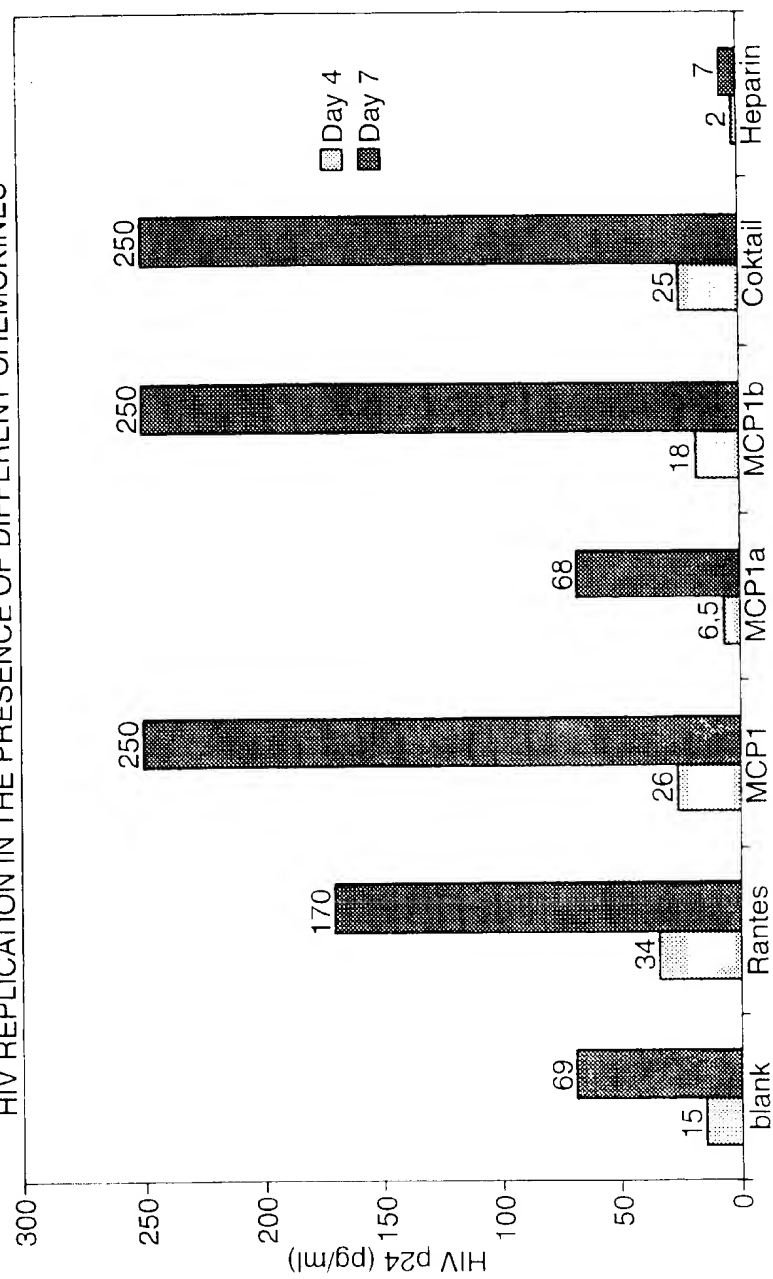
HIV INFECTION OF P4C5 CELLS IN THE  
PRESENCE OF 35K AND SOLUBLE CD4

Fig.16.

EFFECT OF HEPARIN ON PROTEIN  
SYNTHESIS IN MACROPHAGES

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Fig.15.  
HIV REPLICATION IN THE PRESENCE OF DIFFERENT CHEMOKINES



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## Fig.17.

DNA sequence encoding the 35 kDa protein of vaccinia virus strain Lister

1451 AATTAATTTC TCGTAAAAGT AGAAAATATA TTCTAATTTA TTGCACGGTA  
1501 AGGAAGTAGA ATCATAAAGA ACAGTACTCA ATCAATAGCA ATTATGAAAC  
1551 AATATATCGT CCTGGCATGC ATGTGCCTGG CGGCAGCTGC TATGCCTGCC  
1601 AGTCTTCAGC AATCATCCTC ATCCTCCTCC TCGTGTACGG AAGAAGAAAA  
1651 CAAACATCAT ATGGGAATCG ATGTTATTAT CAAAGTCACA AAGCAAGACC  
1701 AAACACCGAC CAATGATAAG ATTTGCCAAT CCGTAACGGA AATTACAGAG  
1751 TCCGAGTCAG ATCCAGATCC CGAGGTGGAA TCAGAAGATG ATTCCACATC  
1801 AGTCGAGGAT GTAGATCCTC CTACCACTTA TTACTCCATC ATCGGTGGAG  
1851 GTCTGAGAAT GAACTTTGGA TTCACCAAAT GTCCTCAGAT TAAATCCATC  
1901 TCAGAATCCG CTGATGGAAA CACAGTGAAT GCTAGATTGT CCAGCGTGTC  
1951 CCCAGGACAA GGTAAGGACT CTCCCGCGAT CACTCGTGAA GAAGCTCTTG  
2001 CTATGATCAA AGACTGTGAG GTGTCTATCG ACATCAGATG TAGCGAAGAA  
2051 GAGAAAGACA GCGACATCAA GACCCATCCA GTACTCGGGT CTAACATCTC  
2101 TCATAAGAAA GTGAGTTACG AAGATATCAT CGGTTCAACG ATCGTCGATA  
2151 CAAAATGTGT CAAGAATCTA GAGTTTAGCG TTCGTATCGG AGACATGTGC  
2201 AAGGAATCAT CTGAACTTGA GGTCAAGGAT GGATTCAAGT ATGTGCACGG  
2251 ATCGGCATCT GAAGGTGCAA CCGATGATAC TTCACTCATC GATTCAACAA  
2301 AACTCAAAGC GTGTGTCTGA ATCGATAACT CTATTCATCT GAAATTGGAT  
2351 GAGTAGGGTT AATCGAACGA TTCAGGCACA CCACGAATTA AAAAAGTGTA  
2401 CCGGACACTA TATTCGGTT TGCAAAACAA AAATGTTCTT AACTACATTC  
2451 ACAAAAAGTT ACCTCTCGCG ACTTCTTCTT TTTCTGTCTC AATAGTGTGA  
2501 TACGATTATG AACTATTCCT TATTCCTATT CCTATTTCTT TTCAGGGTAT  
2551 CACAAAAATA TTAAACCTCT TTCTGAT



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## Fig.18.

Amino acid sequence of 35 kDa protein of vaccinia virus strain Lister

```
1  MKQYIVLACM CLAAAAMPAS LQQSSSSSSS CTEENKHHM GIDVIAKVTK
51  QDQTPTNDKI CQSVTEITES ESDPDPEVES EDDSTSVEDV DPPTTYYSII
101 GGGLRMNFGF TKCPQIKSIS ESADGNTVNA RLSSVSPGQG KDSPAITREE
151 ALAMIKDCEV SIDIRCSEEE KDSDIKTHPV LGSNISHKKV SYEDIIGSTI
201 VDTKCVKNLE FSVRIGDMCK ESSELEVKDG FKYVDGSASE GATDDTSLID
251 STKLKACV
```

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 98/00571

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/16 //C07K14/065

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARTINEZ-POMARES L ET AL.: "MAPPING AND INVESTIGATING THE ROLE IN PATHOGENESIS OF THE MAJOR UNIQUE SECRETED 35-KDA PROTEIN OF RABBITPOX VIRUS" VIROLOGY, vol. 206, no. 1, 10 January 1995, ORLANDO US, pages 591-600, XP002068214 cited in the application see page 592, right-hand column, paragraph 2  --- -/-	8

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

16 June 1998

Date of mailing of the international search report

07.07.98

Name and mailing address of the ISA

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Authorized officer

Cupido, M

## INTERNATIONAL SEARCH REPORT

Inter:      nal Application No  
PCT/GB 98/00571

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D'SOUZA M P ET AL: "CHEMOKINES AND HIV-1 SECOND RECEPTORS" NATURE MEDICINE., vol. 2, no. 12, December 1996, CO US, pages 1293-1300, XP002068215 cited in the application see page 1298, right-hand column, paragraph 3 ---	1-7
A	ARENZANA-SEISDEDOS F ET AL: "HIV BLOCKED BY CHEMOKINE ANTAGONIST" NATURE, vol. 383, 3 October 1996, page 400 XP002025860 ---	1-7
P,X	ALCAMI A ET AL.: "BLOCKADE OF CHEMOKINE ACTIVITY BY A SOLUBLE CHEMOKINE BINDING PROTEIN FROM VACCINIA VIRUS" JOURNAL OF IMMUNOLOGY., vol. 160, no. 2, 15 January 1998, BALTIMORE US, pages 624-633, XP002068216 cited in the application see page 632, right-hand column, last two paragraphs -----	1-7

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 98/00571

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 1-4, insofar as an in vivo method is concerned, are directed to a method of treatment of the human or animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.